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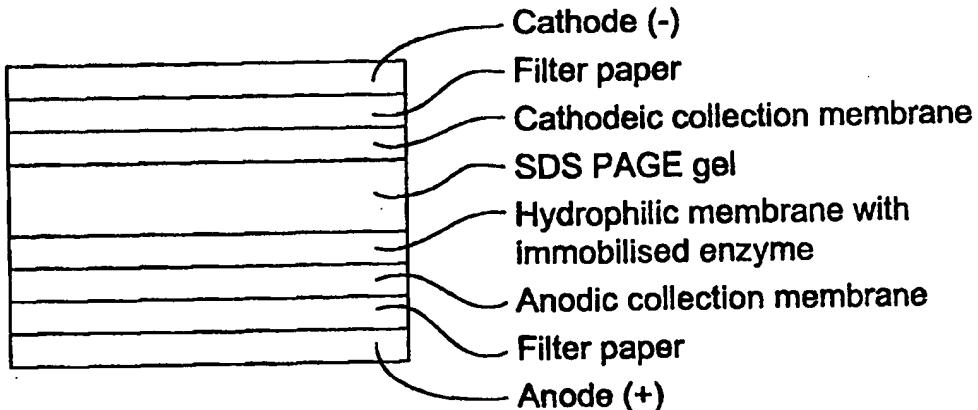


(57) Abstract

Polypeptides which have been separated by gel electrophoresis can be identified or characterised by a procedure which has two main stages. In the first stage the gel is digested with a polypeptide–cleaving agent such as an enzyme. This produces mainly large fragments which, in the second stage are electroblotted through a hydrophilic membrane on which is immobilised another polypeptide–cleaving reagent such as an enzyme onto a hydrophobic member, typically a membrane, e.g. of PVDF. The resulting fragments, usually peptides, are identified, preferably by MALDI–TOF MS, or a property may be determined, e.g. by interaction with an antibody.

## "METHOD AND KIT FOR IDENTIFYING OR CHARACTERISING POLYPEPTIDES"

### Background of the invention

5      ### 1. Field of the invention

This invention relates to the identification or characterisation of one or more polypeptides which have been isolated on a gel, typically from polyacrylamide gel electrophoresis (PAGE) and to a kit for use in the

10     method. It is especially useful in proteomics (the large scale identification and characterisation of proteins).

### 2. Description of the related art

In proteomics, massively parallel protein identification and characterisation techniques are

15     required. The identification of proteins or other polypeptides merely by PAGE, even using two-dimensional gels (2D-PAGE), is laborious and often uncertain. Many different methods have been developed to identify and partially characterise proteins from complex biological

20     samples. Some of them use Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) techniques to analyse peptide "fingerprints" produced by fragmenting the proteins with enzymes. Several software programs have been developed to

25     compare mass spectra of the peptides obtained from MALDI-TOF MS experiments with theoretical spectra from proteins. The subject has been reviewed by M. Kussmann and P. Roepstorff, Spectroscopy 1998, *14*, 1-27. These authors noted three ways in which proteins separated by

30     gel electrophoresis could be digested with enzymes to yield fragment peptides:

1. The digestion can be carried out in a plug of excised gel and the peptides recovered by elution. This is the authors' own preference.

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2. The protein can be first electroleluted from an excised gel plug and then digested in solution.

3. The protein can be electroblotted onto a membrane and subsequently digested on the membrane.

These types of processes are not practical for the sequencing of polypeptides which have been run on the same gel, since the cutting out of the polypeptide bands from the gel has to be done sequentially and the plugs thus obtained placed in tubes for further analysis. Also, losses occur when the polypeptides adhere to the walls of the tube.

Two of the present inventors have experimented with a different method, which they have termed one-step digestion transfer (OSDT). See US Patent Application SN 09/107 991 filed June 30, 1998 and corresponding Canadian Patent Application No. 2 244 947 filed September 24, 1998 entitled "Methods of identifying polypeptides", the disclosure of which is herein incorporated by reference. They have found that the proteins or other polypeptides separated on a gel can be cleaved into fragments, for example by digestion with an enzyme, and that these fragments are presented very satisfactorily for analysis, especially by MALDI-TOF MS, if the cleaving reagent is immobilised on a hydrophilic membrane and interposed as the "filling" in a blotting "sandwich" between the separation gel as one "slice" of the sandwich and a hydrophobic collection member, exemplified as a conventional polyvinylidene fluoride (PVDF) membrane, as the other "slice" of the sandwich. In this way, the fragments are collected on the hydrophobic member and can then be formulated in an appropriate way for the MALDI-TOF MS. It is only necessary that the transblotting is carried out so that the proteins have a long enough residence period in the proximity of the immobilised

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cleaving reagent to ensure that a reasonable amount of
the fragments is produced, but, of course, not so long as
to allow undesired diffusion. With electroblotting, i.e.
blotting assisted by an electric field, this is easily
5    achievable by varying appropriately the current used in
the electroblotting, e.g. by pulsing the current or using
a unsymmetrical alternating current. Further, when an
enzyme is used as the cleaving agent and when the enzyme
is immobilised securely on a hydrophilic membrane,
10   especially by covalent bonding to the solid phase,
autodigestion (cleavage of the enzyme by itself) is
inhibited.

        The OSDT method gives good results for many
proteins, but very strongly basic proteins such as
15   lysozyme are not easily transferred under the conditions
which are optimal for use of the preferred enzyme,
trypsin. Trypsin gives best digestion in a buffer of pH
about 8.4. Also, the OSDT method does not give good
digestion of very high molecular weight proteins.

20   Summary of the Invention
        The present invention is an improvement to the OSDT
method and is based in part on the discovery of another
technology which the inventors have termed "in full gel
digestion" (IFG). This procedure involves dehydrating
25   the gel and then rehydrating it, adding to gel a
polypeptide-cleaving reagent such as an enzyme, e.g. in
the rehydration buffer. After the IFG, the digested
proteins are then electroblotted in a conventional way.
One drawback of this technique is the loss of low
30   molecular weight proteins (those of m.w. less than 40kDa)
by diffusion during the in-gel digestions.

        It has now been found that by combining the IFG
procedure, optionally modified, with OSDT, satisfactory
digestion of the proteins (or other polypeptides),

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accompanied by improved identification, can be achieved for polypeptides having a wide range of molecular weights. Moreover, high molecular weight proteins can be satisfactorily immunoblotted to yield fragments which can
5    be identified as epitopes.

In a preferred "combined procedure", the gel is dehydrated and at least partially, preferably only partially, rehydrated with a buffer containing the polypeptide-cleaving reagent, IFG is performed and this
10   is then followed by OSDT.

In one aspect the invention provides a method of identifying or characterising polypeptides which have been isolated on a gel by electrophoresis, comprising:

a)    providing a gel on which at least one polypeptide
15   has been isolated;

b)    incorporating a first polypeptide-cleaving reagent in the gel (preferably by dehydrating the gel and at least partially rehydrating it with a buffer containing the polypeptide-cleaving reagent);

20   c)    providing adjacent to the gel at least one hydrophilic membrane on which is immobilised at least one second polypeptide-cleaving reagent ;

d)    providing a hydrophobic collection member (preferably a membrane) suitable for receiving thereon
25   fragments of polypeptide transferred thereto from the gel by transblotting, preferably by electroblotting, said hydrophobic member being positioned beyond the hydrophilic membrane in a direction of movement of the fragments of polypeptide;

30   e)    transblotting the polypeptide or polypeptides from the full gel, on which the polypeptide or polypeptides were isolated, through the hydrophilic membrane or membranes, under conditions effective to cause it or them

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to be cleaved into fragments by the second polypeptide-
cleaving reagent, to the hydrophobic member; and

f) identifying or characterising the fragments
collected on the hydrophobic collection layer.

Preferably the method further comprises

g) identifying or characterising the polypeptide from
which the fragments were derived.

The invention also includes a kit for use in the
method of the invention, said kit comprising:

a) a first polypeptide-cleaving reagent suitable for
incorporating in an electrophoretic gel;

b) at least one hydrophilic membrane suitable for use
in transblotting of polypeptides separated on an
electrophoretic gel, the membrane having at least one
second polypeptide-cleaving reagent immobilised thereon;
and

c) a hydrophobic collection member suitable for
receiving thereon fragments of the separated polypeptides
transferred thereto by transblotting.

Elements b) and c) may be provided as separate
components, e.g. in separate containers, or as a pre-
formed assembly.

The term "cleaving a polypeptide", as used herein,
refers to any step in which a group, residue or any chain
of groups or residues is split off from the remainder of
the molecule. It includes cleavage in the main chain of
amino acids or in a side-chain or of any terminal or
side-chain group or residue, e.g. removal of a C-terminal
amino acid by carboxypeptidase, N-terminal amino group by
an aminopeptidase or a glycosyl side-chain by a
glycosidase is included.

The method of the invention requires digestion in
the full gel. That is, the method does not include

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cutting pieces from the gel and digesting the cut pieces in an enzyme.

Reference above to the gel having been dehydrated covers allowing it to become dehydrated merely by standing in air at ambient temperature or taking deliberate steps to dehydrate it. The term "dehydration" includes complete, substantially complete or partial removal of water from the gel.

The term "collection member" as used herein has a broad meaning, since this is not in itself critical to the invention. Considered in isolation, it may be, for example, a self-supporting membrane, film, or plate, or it may be non-self-supporting, e.g. a hydrophobic layer supported on a substrate, e.g. as a coating. It will normally be porous to the blotting buffer, to enable current to be carried to or from the electrode, but may alternatively be the electrode or in direct electrical communication with it.

The term "transblotting", as used herein, covers any operation of transferring the polypeptide fragments to another surface, which, in this invention, is a hydrophobic collection member. It includes a process of transfer by capillary action or by electroblotting. In this specification, "transblotting" can be part of any blotting procedure applicable to polypeptides, including, for example immunoblotting.

The term "identifying" as used herein is not synonymous with determining the sequence and includes partially identifying the polypeptide. Further, it includes making a tentative identification based on the most probable of a small number of possibilities.

The term "kit" as used herein includes combinations of the identified components in separate containers and also an assembly of the hydrophilic membrane(s) and

hydrophobic collection layer ready for use. The kit may further include, in separate containers, other reagents useful in the method of the invention, e.g. a buffer for rehydrating the gel, a blotting buffer, reagent(s) which

5   assist in the reaction of the enzyme with the polypeptide fragment and so on.

Brief description of the drawings

Figs. 1 and 2 are schematic views of two kinds of blotting "sandwich" which can be used in the invention;

10  Fig. 3 is a plot of applied voltage against time, showing the production of an alternating voltage for use in electroblotting in the method of the invention;

Figs. 4A-4D and 5A-5D show stained polypeptide bands present on the collection membrane to which the proteins

15  and protein fragments have been transferred, respectively, for control (4A, 5A), IFG digestion (4B, 5B), OSDT (4C, 5C) and the combined method of the invention (4D, 5D); and

Figs. 6A-6C and 7A-7C show the MALDI-TOF MS spectra

20  obtained from myosin and chicken lysozyme respectively, for IFG (6A, 7A), OSDT (6B, 7B) and the combined method of the invention (6C, 7C).

Description of the preferred embodiments

The invention relates to identifying polypeptide(s)

25  which have already been isolated on a gel by gel electrophoresis. The nature of the polypeptide(s) to be identified is not critical. They can be, for example, naturally occurring proteins, proteins made by recombinant DNA technology, polypeptide(s) made by

30  peptide synthesis or by expression of recombinant DNA. For brevity, the invention will be described hereinafter with reference to proteins. The extrapolation to other polypeptide(s) will be taken as understood and incorporated throughout the following description.

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The kind of gel on which the proteins have been isolated is not critical, but will usually be a polyacrylamide gel. Any of the conventional gels and separation conditions may have been employed, including reducing conditions. They may be one-dimensional or two-dimensional gels. (In 2D gels, proteins etc. are separated in one dimension by their charge and in the other dimension by their molecular mass).

The invention is normally to be applied to multiple proteins co-present on the same gel, for example from 3 to 3000, more usually 30 to 3000 and preferably 50 to 1500, proteins. This includes proteins present at different molecular weight separations on a 1D gel or at similar molecular weight separations, but present in parallel lanes or tracks on the 1D gel, as well as those separated by 2D gel electrophoresis. However, the invention also applies to a gel on which a single polypeptide is required to be identified or characterised. In particular, it is useful in relation to immunoblotting of proteins of high molecular weight, e.g. 150-250kDa, in order to split them into fragments in which epitopes can be recognised by immunoblotting.

The first stage of the method of the invention comprises digestion of the proteins within the same gel as that on which they were separated. Within this stage, there are preferably three main operations. The first operation is to dehydrate the gel. The gel may be fully or partially dehydrated depending on the amount of protein digestion required. The greater the dehydration the greater the capacity of the gel subsequently to absorb a solution containing the first polypeptide-cleaving reagent and therefore the greater the digestion. Dehydration may be complete, substantially complete, e.g. by removal of 90-99% by weight of the original water

content, or, more preferably, partial, e.g. by removal of 25-90%, preferably 40-70%, on the same basis. Of course, it is more troublesome to rehydrate a completely dried-out gel. The method of dehydration is not critical. Air-drying at ambient temperature, say 15-25°C is preferred. Mere standing of the gel at 4-10°C under low pressure is another method. Air-drying, followed by standing, is a further method.

The next operations are rehydration and the incorporation of the first polypeptide-cleaving reagent in the rehydration buffer. The polypeptide-cleaving reagent is preferably an enzyme such as trypsin or Lys-C, but a chemical cleaving agent such as CNBr could alternatively be used. Examples of other enzymes and chemical cleaving reagents are given later, in connection with the discussion of the second polypeptide-cleaving reagent. Hereinafter enzymes will be referred to, the extrapolation to other polypeptide-cleaving reagents being taken as understood and incorporated *mutatis mutandis* in the following description. The first enzyme may be the same as or different from the second enzyme(s) used in the transblotting step. Any of the enzymes described below for use in transblotting can be used in the in full gel digestion.

In principle, it is immaterial at what stage the first enzyme is introduced into the gel and whether it is present in the gel in a free or immobilised form. However, if present in the gel from the start of electrophoresis, it would normally upset the pattern of protein separation, unless the enzyme or conditions of running the gel were so chosen as to make it inactive while the gel is being run. This could be achieved by adding a reversible inhibitor, of the enzyme to the running buffer. For example, if the enzyme is trypsin a

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reversible inhibitor such as benzamidine would be suitable. Conditions are e.g. as described in S. L. Jeffcoate *et al.*, J. Clin. Endocrinal. Metab. 1974 *38*, 155-157. Then, after the gel has been run, resulting in
5   separation of proteins, it is washed as part of the rehydration step, resulting in the removal of inhibitor and therefore re-activation of the enzyme.

The enzyme is normally added to the gel after isolation of the proteins. It is added as a solution or
10  fine suspension which will penetrate the gel and be absorbed well by the gel solids. In principle, the enzyme could be added before any deliberate step of rehydration or even after rehydration has been effected, e.g. in a concentrated solution. However, in practice,
15  such techniques are unlikely to give the best digestion. Normally, the enzyme will be present in the rehydration solution, which is buffered. Preferably the gel is incubated with the rehydration buffer containing the enzyme, for example for 30 minutes at 35°C. The time and
20  temperature can be varied, according to the size of fragments desired. Rehydration may be partial, complete or even to an extent in which the gel contains more water than when the proteins were run on it. Any extent of rehydration appropriate to allow transblotting is
25  permissible. In practice, it is convenient to treat the gel with excess rehydration solution and to remove the excess. Otherwise, undesired protein diffusion or excessive swelling of the gel can occur.

At this stage the gel contains peptides with a high
30  number of missed cleavages, from partially digested proteins. The fragments of high molecular weight and basic proteins are more easily extractable from the gel by electroblotting, compared with the full molecule.

The second stage of the method of the invention comprises transblotting, preferably electroblotting. Normally, the electroblotting takes place overall in the direction cathode to anode, as the proteins are negatively charged. Depending on the pH of the electroblotting buffer used, positively and negatively charged fragments could be obtained and migrate in opposite directions, towards the cathode and anode respectively. Figures 1 and 2 of the drawings exemplify some sandwiches for the electroblotting. Figure 1 shows an experimental arrangement in which a cathodic collection layer, which is preferably a conventional PVDF membrane, was provided, just to show that under these conditions no proteins migrated to this membrane, despite the alternating field applied (thus reversing the electrodes). It will be understood that under different pH conditions, some fragments could be produced at the cathodic collection layer. Thus, the invention includes the possibility of providing anodic and cathodic collection layers, with hydrophilic membranes interposed between each of them and the separation gel layer. In Figure 1 there is a single hydrophilic membrane, which is preferably a modified PVDF membrane, having an appropriate protein-cleaving reagent, normally a protease enzyme, for example trypsin, immobilised on it, interposed between the gel layer and an anodic collection layer, most conveniently a conventional PVDF membrane, on which the protein fragments are collected. In Figure 2 there is no cathodic collection layer, but there are two consecutive hydrophilic membranes, preferably modified PVDF membranes, each with trypsin immobilised thereon, placed between the gel layer and the anodic collection layer, which, again, is preferably a conventional PVDF membrane.

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In more detail, the anode and cathode are separated from the rest of the sandwich by an absorptive layer which soaks up the blotting liquid, while maintaining the liquid in electrical contact with the electrodes, and is
5    conveniently a filter paper.  The kinds of electrodes and absorptive layers used in arrangement are not critical and can be any conventionally used in electroblotting.

The anodic collection layer (and cathodic collection layer if used) are also not critical and thus can be any
10   conventional   hydrophobic   membrane   used   in electroblotting, such as PVDF (conventional or positively charged), nylon or nitrocellulose, for example.

The "filling" of the sandwich can take the form of one or more  membranes (defined as above) sufficiently
15   hydrophilic in character that the proteins and fragments thereof do not tend to stick thereon.  This membrane can be formed from any thin member which is porous to the electroblotting liquid and capable of immobilising the polypeptide-cleaving reagent thereon, whether on the
20   surface thereof of within interstices or microcavities therein accessible to the electroblotting liquid (and therefore to the polypeptide to be cleaved).  It will typically be from 100 to 600 µm thick.  Usually the number of such membranes will be from 1 to 3.  With conventional
25   thicknesses of membrane, e.g. 130 to 150 µm as in the preferred "Immobilon AV" PVDF membrane, 2 membranes will frequently be used.  They are best placed directly mutually  adjacent,  i.e.  one  on  top  of  another. Preferably, the second polypeptide cleaving reagent is
30   bonded to the hydrophilic membrane covalently.  For this purpose, the hydrophilic membrane(s) are preferably provided with "active carbonyl" or carboxylic acid groups or derivatives thereof reactive with amino groups present in the cleaving reagent, e.g. an enzyme.  "Active

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carbonyl"-modified or carboxyl-modified PVDF membranes
are especially preferred.

Since it would be difficult to react all the active
groups present on the surface of a membrane with an
5      enzyme, and since it is undesirable to allow the
polypeptides to react with these free active groups, the
residual active groups (which would otherwise be free)
are preferably capped before the membrane is used, e.g.
with ethanolamine, thus providing terminations such as   -
10     $CO-NH-CH_2-CH_2-OH$, which are relatively hydrophilic. Other
hydrophilic capping groups will suggest themselves to
those skilled in the art.

Alternatively, PVDF membranes or glass fibre paper
can be functionalised by isothiocyanate, which allows
15     reaction with the N-terminal amino groups and/or the $\varepsilon$-
amino groups of lysine residues in the enzymes. For this
purpose, the PVDF membranes are pre-treated with NaOH to
provide a carbon-carbon ethylenic double bond in the
polymer chain, by elimination of a molecule of HF. The
20     pre-treated PVDF membranes are then reacted under basic
conditions with a dinucleophile such as ethylenediamine,
1,10-diaminodecane    or    2-aminoethanethiol,    whereby
hydrogen atoms in the polymer are substituted by   -X-
$(CH_2)_n-NH_2$ groups, wherein -X- is -S- or -NH- and n is 2
25     or 10. This polymer, having amine-terminated side-
chains,        is      then      reacted      with     1,4-
phenylenediisothiocyanate (DITC) or 3,5-dichloro-1,4-
phenylenediisocyanate (DCDITC) to give the required
isothiocyanate-terminated side-chains in good yield.
30     DITC-reacted glass fibre sheets provide another form of
membrane, see R.H. Aebersold et al., J. Biol. Chem. 1986,
*291*, 4229-4338.

Another form of hydrophilic membrane is PVDF
functionalised by arylamine groups, which react with a

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carboxylic acid side-chain or the carboxyl terminus of the enzyme, preferably in the presence of a carbodiimide such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.

Another form of hydrophilic membrane which can be used as the sandwich filling is a thin film or coating of agarose gel. The N-terminal amino and/or ε-amino groups (according to the selectivity of the reaction) of lysine residues in the enzyme are treated to obtain aminoxy groups, which react with aldehyde groups produced by mild oxidation of the agarose gel, thus bonding the enzyme covalently to the agaraose.

A further kind of hydrophilic membrane may comprise one or more thin films or coatings of polyacrylamide gel, similar in thickness to that used in immobilised pH gradient electrophoresis (IPG), but which has been trypsinated. This can be done by reacting trypsin with acryloyl chloride to form an N-acryloyltrypsin, which is then copolymerised with acrylamide in the preparation of an acrylamide copolymer gel.

Yet another form of hydrophilic membrane is a glass fibre paper which has been modified to replace amino groups by groups containing a diazo linkage, e.g. 4-N,N-dimethylaminoazobenzene-4'-isocyanate groups. The reactions required for this purpose have been described by J.Y. Chang et al., FEBS Letters 1977, 84, 187-190.

The cleaving reagent immobilised on the membrane is normally and preferably immobilised by covalent bonding. However, other forms of immobilisation are not excluded from use in this invention, so long as the enzyme does not become sufficiently free in solution in the electroblotting liquid as to undergo autodigestion. (It will be understood that the presence of autodigested enzyme fragments could upset the analysis of the fragments from the protein to be analysed). Thus, for

example, the enzyme could be physically trapped within the pores of a porous sheet of hydrophilic polymer. Alternatively, the membrane could have an enzyme immobilised thereon by means comprising (consisting of or

5   including) affinity bonding. Thus, the enzyme could be covalently attached to avidin or streptavidin and the resultant conjugate attached to a biotinylated membrane by affinity bonding between avidin/streptavidin and biotin. Alternatively, avidin or streptavidin could be

10  attached to the membrane and the enzyme could be reacted to provide biotinyl terminations for reaction with a membrane to which avidin or streptavidin has been attached.

Preferably either or both polypeptide-cleaving

15  reagents comprise an enzyme. If both comprise an enzyme, it may be the same or different. Most preferably and usually, the enzyme cleaves the main chain of the polypeptide (i.e. is an endopeptidase or endoproteinase), especially trypsin. Trypsin cuts proteins at the C-

20  terminal end of many lysines and arginines. Other less specific endoproteases, e.g. pepsin or such as chymotrypsin are usable, as are highly specific enzymes such as Lys-C, Arg-C or Glu-C. For phosphoproteins, a phosphorylase is useful. Either or both enzymes can be

25  an exo-enzyme which splits off a side-chain of the protein or acts at the terminus. More than one enzyme can be incorporated in the gel. More than one enzyme can be immobilised on the membrane. For example, it may be helpful to split off one or more side chains of the

30  polypeptide, e.g. using a carboxypeptidase or aminopeptidase, in conjunction with an endoproteinase. Carboxypeptidase Y is one particularly useful such enzyme.

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To investigate the presence of side-chains in proteins, such as glucosyl, N-acetyl-O-glucosaminyl and sialyl, enzymes which will cleave those chains, such as glucosidase, N-acetylglucosaminidase and neuraminidase, respectively, are useful in the invention.

The following chart indicates the various possibilities for types of enzymes which may be used in combination:

| IFG | OSDT |
|---|---|
| Endopeptidase, e.g. trypsin | Endopeptidase, e.g. trypsin |
| Endopeptidase, e.g. trypsin | Exo-enzyme, e.g. glucosidase, phosphorylase |
| Exo-enzyme, e.g. glucosidase, phosphorylase | Endopeptidase, e.g. trypsin |
| Exo-enzyme, e.g. glucosidase, phosphorylase | Exo-enzyme, e.g. glucosidase, phosphorylase |

The cleaving reagents are not confined to enzymes. Either or both can be a chemical reagent, for example cyanogen bromide, 2-iodosobenzoic acid or a derivative thereof or hydroxylamine. Such reagents are described by E.A.Carrey, "Peptide Mapping" in "Protein Structure: A Practical Approach", ed. T.E.Creighton, IRL Press, 1989, pages 117-121. For use as the second polypeptide-cleaving agent, the chemical reagents are suitably immobilised on the hydrophobic member. Thus, cyanogen bromide can be physically immobilised by entrapment within pores of the hydrophilic membrane. 2-Iodosobenzoic acid can be derivatised, preferably at the COOH group, e.g. with an alkylenediamine, especially 1,6-hexanediamine, leaving a free amino group which is then reacted with functional groups on the membrane, such as active carboxyl groups mentioned above.

It will be appreciated that different cleaving reagents will have different specificities and in certain

cases the absence of small fragments, indicating the absence of cleavage, may be a useful result for identification or characterisation.

The electrical current applied in the electroblotting is preferably not a direct, continuous current, but either pulsed, i.e. a direct current with intervals in which no current is passed, or an alternating current. It may be unbiased or biased in the cathode to anode direction, i.e. mainly a cathode to anode current, but with intervals in which current is passed in the opposite direction. Variations on these regimes are possible within the general spirit of the idea of performing a slower than normal electroblotting, allowing sufficient time for the cleavage to take place on the hydrophilic membrane(s), while avoiding such a slow journey of the protein fragments from the separation gel to the collection membrane that lateral diffusion occurs, causing loss of resolution.

The electroblotting liquid is not critical to obtaining some form of protein fragmentation and hence a useful identification. It is normally buffered and can be any conventional buffer for this purpose, such as Tris/glycine with methanol or 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) with methanol. The direction of migration of the fragments depends essentially on the pH of the buffer. For most purposes an alkaline buffer will be appropriate, since many enzymes function best at alkaline pH. Particularly, in the case of trypsin, the buffer will preferably have a pH from 8.0 to 9.0 and most especially 8.2 to 8.6, with a half Towbin's buffer of pH about 8.4 being considered optimal. Some other enzymes, such as endoproteinase V8, require an acidic pH. Under such conditions, the fragments will migrate to the cathode.

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It has been found desirable to incorporate a small amount of a conventional detergent such as SDS in the buffer, to produce micelles (negatively charged aggregates).

5      The protein fragments, whether they are peptides derived from the main chain of the protein or are residues of a side-chain, are collected on the collection layer.   They are then preferably analysed by a spectroscopic method based on matrix-assisted laser

10    desorption/ionisation (MALDI) or electrospray ionisation (ESI).   The preferred procedure is MALDI with time of flight (TOF) analysis, known as MALDI-TOF MS.   This involves forming a matrix on the membrane, e.g. as described in the literature, with an agent which absorbs

15    the incident light strongly at the particular wavelength employed. The sample is excited by UV, or IR laser light into the vapour phase in the MALDI mass spectrometer. Ions are generated by the vaporisation and form an ion plume.   The ions are accelerated in an electric field and

20    separated according to their time of travel along a given distance, giving a mass/charge (m/z) reading which is very accurate and sensitive. MALDI spectrometers are commercially available from PerSeptive Biosystems, Inc. (Framingham, MA, USA) and are described in the

25    literature, e.g. M.Kussmann and P. Roepstorff, cited above.

In this invention, the above method is applied to the scanning of the fragments of many proteins at once. Thus, many proteins can be run simultaneously on a

30    polyacrylamide gel, subjected to the method of the invention to produce an array of spots on the collecting membrane and the array analysed as follows. After the PVDF membrane or other hydrophobic collection layer has been stained, a piece of it will be cut and fixed on the

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MALDI-MS sample plate, e.g. with silicon grease. An organic matrix-forming reagent is added to the membrane on the sample plate and the sample is then air-dried to form the matrix. The sample plate is inserted in the
5    MALDI-MS spectrometer. An automated movement of the sample plate from a first to a second position and subsequent positions, to align the laser with individual spots in the array, is arranged by computer program. At each position a MALDI-MS spectrum is generated, the
10   spectral information collected in digital form and the data downloaded to the ExPASy database research program (PeptIdent).

It is then relatively simple to provide automated output of the results by using the ExPASy server, as at
15   present used for MALDI-TOF MS and to generate the data in a form handleable by computers.

It will be evident, therefore, that the present invention has huge potential for the automated identification and/or partial characterisation of
20   proteins, e.g. in proteomics research. In effect, the invention provides in this preferred embodiment a "molecular scanner" for this purpose.

Other techniques for improving the mass accuracy and sensitivity of the MALDI-TOF MS can be used to analyse
25   the fragments of protein obtained on the collection membrane. These include the use of delayed ion extraction, energy reflectors and ion-trap modules. In addition, post source decay and MS-MS analysis are useful to provide further structural analysis. With ESI, the
30   sample is in the liquid phase and the analysis can be by ion-trap, TOF, single quadrupole or multi-quadrupole mass spectrometers. The use of such devices (other than a single quadrupole) allows MS-MS or $MS^n$ analysis to be performed.

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Still other methods of analysis comprise immunoblotting using monoclonal or polyclonal antibodies and phospho-imaging.

Optionally, other components may be included in the
5    kit, especially any one or more of the following: matrix-forming reagent for MALDI-TOF, rehydration buffer, electroblotting buffer, collection layer(s), e.g. cationic membranes and PAGE materials. In the case of immunoblotting, the kit may further comprise one or more
10   antibodies, especially a range of monoclonal antibodies. Kits as defined above, but further comprising any one, two or more of the above optional components are hereby specifically declared to be within the invention. All components of the kit may be supplied in separate
15   containers, but packaged overall as a kit.

The following Examples illustrate the invention. The words "Immobilon", "Trans-Blot", "Trizma", "Tween" and "Voyager" are Trade Marks and/or Registered Trade Marks.

20   <u>EXAMPLES</u>
<u>Materials and methods</u>

*Chemicals.* "Immobilon" type AV (IAV) membranes were purchased from Millipore (Bedford, MA, USA). Acrylogel-PIP 2.6%C solution was purchased from BDH (Poole,
25   England). Broad range SDS-PAGE standard PVDF membranes were purchased from Bio-Rad (Richmond, CA, USA). Trifluoroacetic acid (TFA), "Trizma base" (Tris), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and trypsin (type IX from porcine pancreas, dialysed and
30   lyophilised) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (preparative HPLC grade), calcium chloride, ethanolamine, glycine and $\alpha$-tosyl-L-arginine methyl ester (TAME) were purchased from Fluka (Buch, Switzerland).

12.5%, 2.6% C linear gel (home made). To produce a
12.5% T, 2.6% C linear polyacrylamide gel, crosslinked
with PIP ("PIP" = N,N'-diacryloylpiperazine), 8 ml of
Acrylogel-PIP 2.6%C stock solution were mixed with 5 ml

5      of Tris-HCl 1.5 M pH 8.8 and 6.6 ml of deionised water.
The polymerisation of the gel was induced with 20 µl of
TEMED and 100 µl of APS (10% w/v). The solution was
degassed and loaded into a Bio-Rad mini-2D gel support.
To preserve the gel from the atmosphere, 0.5 ml of water-

10     saturated sec-butanol was added on the top of the gel.
After 30 minutes, the gel was washed for subsequent
loading of the 4% stacking gel. It was obtained from the
mixture of 2.6 ml Acrylogel-PIP 2.6%C stock solution, 5
ml of Tris-HCl 1.5 M, pH 8.8, 12.3 ml of deionised water,

15     20 µl of TEMED and 100 µl of APS (10% w/v). The solution
was degassed and loaded on top of the gel. A comb was
inserted before gel polymerisation to create 15 sample
wells. The gel can be used directly after 30 minutes of
polymerisation.

20     *1-D PAGE.* For the 1-D PAGE method, Mini-Protean II
electrophoresis apparatus (Bio-Rad, Richmond, CA, USA)
was used. SDS-PAGE was conducted essentially according to
the method of Laemmli, with 12.5%T, 2.6%C polyacrylamide
gel. The protein samples used were Bio-Rad SDS-PAGE

25     standards. They were bovine pancreatic trypsin inhibitor
(6.5 kDa), chicken lysozyme (14.3 kDa), soybean trypsin
inhibitor (20.1 kDa), bovine carbonic anhydrase (28.9
kDa), chicken ovalbumin (42.7 kDa), bovine serum albumin
(66.4 kDa), and rabbit phosphorylase b (97.2 kDa),

30     *E.coli.* β-galactosidase (116.4 kDa) and rabbit myosin
(about 200 kDa). Protein migration was carried out on a
single lane at 200 V for 40-50 minutes.

*Covalent attachment of trypsin and blockage of the
IAV membrane.* IAV membrane is a commercially available

modified PVDF membrane, having activated carboxylated groups. These groups are reactive towards nucleophiles such as amine groups from proteins or peptides. Based on the above-cited Millipore technical documentation on "Immobilon AV", trypsin was immobilised on this membrane (Figure 1).

A 10 x 12 cm IAV membrane was incubated in a rotating hybridiser HB-2D (Techne, Cambridge, England) with 20 ml of a 2.5 mg/ml trypsin solution in 20 mM sodium dihydrogen phosphate buffer, pH 7.8, at room temperature for 3 hours. Then, the membrane was washed 3 times rapidly and vigorously with 20 ml of PBS-"Tween" 20 solution (20 mM of sodium dihydrogen phosphate, 140 mM sodium chloride and 0.5% "Tween" 20, pH 7.4) to remove unreacted trypsin. The membrane was incubated for 3 hours with 20 ml of 1M ethanolamine in 1M sodium bicarbonate, pH 9.5, at 4°C to block the remaining active carboxyl groups of the membrane. After this capping step, the membrane was washed 3 times rapidly and vigorously with 20 ml of the PBS-"Tween" solution and then twice for 30 minutes with 20 ml of the PBS-"Tween" solution. The membranes were stored at 4°C in a 46 mM Tris-HCl, 1.15 mM calcium chloride, 0.1% sodium azide buffer solution, pH 8.1.

*Activity measurement of the enzyme covalently bound to the IAV membrane.* The tryptic activity of the IAV-Trypsin membrane was determined using the trypsin assay reagent TAME. One to 2 $cm^2$ of the IAV-trypsin membrane was immersed in a mixture of 2.6 ml of 460mM Tris-HCl, 11.5mM calcium chloride, pH 8.1, 0.3 ml of 10 mM TAME solution and 0.1 ml of 1mM HCl solution. After 40 seconds of vigorous stirring, the absorbance of the solution was measured at 247 nm with a UV-Visible spectrophotometer (Ultrospec III, Pharmacia Biotech, Uppsala, Sweden). A

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second measurement was made after 3 minutes of constant stirring. The equivalent amount of free active trypsin per surface unit was calculated from the change per minute in optical absorbance at 247 nm.

5      *In full gel (IFG) protein digestion and conventional electroblotting (for comparison).* Immediately after the SDS-PAGE protein separation, the gels were soaked 3 times in deionised water for 5 minutes to eliminate SDS, glycine and Tris. The entire wet gel or a selected part

10     of it was air dried at room temperature during 8 hours or overnight. The gel was rehydrated and incubated at 35°C for 30 minutes with 3-5 times the initial volume of the gel of a solution of 0.05 mg/ml trypsin in 10 mM Tris-HCl, pH 8.2. The excess of trypsin solution was removed.

15     Then, the gel was incubated for a further 30 minutes at 35°C to complete the digestion. Proteins and peptides contained in the gel were electroblotted onto PVDF membranes using standard procedure with CAPS buffer, pH 11, 0.01% SDS in the tank.

20     *One-step digestion transfer (OSDT) electroblotting (for comparison).* Immediately after the SDS-PAGE protein separation, gels were soaked 3 times in deionised water for 5 minutes, and then equilibrated for 15 minutes in half Towbin's buffer (13 mM Tris, 100 mM glycine, 0.01%

25     SDS. 10% methanol, pH 8.3). Electroblotting was carried out in half Towbin's buffer in a laboratory-made semi-dry apparatus. [Note: the "semi-dry" method is well known, see e.g. P. R. Fausset and H. S. Lu, *Electrophoresis* 1991, *12*, 22-27.] It was carried out for 16 hours at

30     room temperature (21 to 24°C), with a square-shape alternating applied voltage, periodically +12.5 V for 125 ms and -5 V for 125 ms. The shape of the applied voltage is shown in Figure 3, in which voltage is plotted on the

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y-axis and time in milliseconds on the x-axis. The
average effective voltage $U_{eff}$ = 3.75V and is given by the
identity

$U_{eff} = \underline{1}$ x [Integral between 0 and T of Udt]

5              T

where U is the voltage at a particular timepoint, dt is
the change in time between the limits of the integration
and T is the total time of one cycle or period = 250 ms.

To perform the enzymatic digestion of the protein
10     during the electroblotting, a double layer of IAV-trypsin
membrane was placed between the polyacrylamide gel as a
protein source and the PVDF membrane as the collecting
surface to create a transblot-digestion sandwich (Figures
1 and 2).

15     After the electroblotting transfer procedure, the
PVDF collection membranes, i.e. on which the fragments
of digested protein were collected, were washed in
deionised water for 5 to 15 minutes. Proteins remaining
in the gel after the electroblotting were stained with
20     Coomassie Blue R250 (0.1% w/v), methanol (30% v/v) and
acetic acid (10% v/v) for 30 minutes. Gels were destained
by repeated washing with methanol (40% v/v), and acetic
acid (10% v/v) solution. The PVDF collection membranes
were stained with Amido Black (0.5% w/v), isopropanol
25     (25% v/v) and acetic acid (10% v/v) for 1 minute and then
destained by repeated washing with deionised water. The
membranes were air-dried before optical scanning.

IFG/OSDT electroblotting (combined method of the
invention). In the combined method, the IFG procedure
30     was modified by allowing only the first 30 minutes for
rehydration and partial protein digestion. The gel was
then transblotted onto PVDF membrane using the OSDT
process described above. The electroblot transfer was

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carried out in half Towbin's buffer containing 0.01% SDS, as described above.

*MALDI-TOF equipment and experimental conditions.*

PVDF membranes were analysed with a MALDI-TOF mass spectrometer "Voyager" Elite (PerSeptive Biosystems, Framingham MA, USA) equipped with 337 nm nitrogen laser. The analyser was used in the reflectron mode at an accelerating voltage of 18 kV, a delay of ion extraction of 100 ns and a low mass gate fixed at 850 Da. Laser power was set slightly above threshold for molecular ion production. Spectra were obtained by summation of 10 to 256 consecutive laser shots without any smoothing procedure. Small pieces of the PVDF (1 x 3 mm square) containing the stained protein were cut from the PVDF collection membrane and fixed on an adaptable sample MALDI plate with silicon grease. For deposition of the matrix required for MALDI-TOF MS, 1 µl of 4 mg/ml α-cyano-4-hydroxycinnamic acid in 30% acetonitrile, 0.1% TFA solution was added to the anodic PVDF membrane. For the internal calibration, the matrix solution contained 20 nM and 100 nM respectively of two C-amidated synthetic peptides of molecular weights 1498.82 Da and 2095.08 Da.

*Treatment of spectra and use of software.*

Detected peaks were submitted to the peptide mass fingerprint search tool "PeptIdent" available on the World Wide Web (http://www.expasy.ch/www/tools.html) located at the ExPASy server (http://www.expasy.ch). No pI limits were introduced to restrict the search. The apparent migration masses given by the Bio-Rad technical information sheet were used as a restricted condition on the mass value with an error of 20%, as well as the specie origin of the protein. Mass tolerance was ±0.2 Da.

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Fragment masses were submitted to "FindMod" (http://www.expasy.ch/www/tools.html) to identify the amino acid sequence of fragments from their spectral molecular weights. FindMod has options to take into account possible cysteine and methionine modifications of proteins.

Results

Trypsin was attached covalently to IAV membranes with a surface enzyme density, as determined by TAME test, of 0.6 to 1.2 µg of active trypsin per $cm^2$. The activity of the trypsin-bound IAV membranes remained stable when they were stored in the Tris-HCl/$CaCl_2$/$NaN_3$ solution at 4°C for periods up to a month. Tryptic activity decreased slightly after use of the membrane in the method of the invention, but not sufficiently to impair its re-use in another experiment.

After SDS-PAGE separation, the nine proteins specified above, were run on the same track in the SDS-PAGE, were subjected to a control electroblotting plus three different digestion techniques:

-       Control electroblotting of the proteins in the tank method, without digestion, using the standard CAPS buffer, pH 11,

-       IFG digestion followed by electrotransfer using the tank method with CAPS buffer,

-       OSDT digestion through IAV membrane using a semi-dry method and the half Towbin's buffer, pH 8.3,

-       Combined method of the invention (partial IFG followed by OSDT).

Figures 4A to 4D show the transblot pattern of the four Amido Black stained PVDF membranes corresponding to a control without digestion (A), IFG digestion (B), OSDT (C) and the combined method of the invention (D), with the molecular weights of the nine proteins indicated.

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Figures 5A to 5D show the pattern of the proteins remaining on the SDS gel.

First, protein transfer using OSDT with half Towbin's buffer, 0.01% SDS, pH 8.4 (Figure 4C) can be compared with the standard transfer using CAPS buffer, 0.01% SDS, pH 11 (Figure 4A). Despite the addition of SDS in the ½ Towbin's buffer, basic proteins such as pancreatic trypsin inhibitor (pI 9.2) and lysozyme (pI 9.3) did not transfer. The influence of the SDS in the buffer was more noticeable for higher molecular weight proteins such as phosphorylase b (97.2 kDa) and myosin (≈200 kDa) (Figures 4A and 4C). However, large amounts of these proteins remained in the gel after the electroblot (Figures 5A and 5C).

In the case of the IFG digestion (Figure 4B) and the combined method (Figure 4D), the PVDF membrane exhibited all of the 9 protein bands. PVDF patterns were similar to the normal transfer (Figure 4A). The major difference between these PVDF membranes was that the intensity of the polypeptide beads was higher for normal protein transfer (Figure 4A) and lower for the combined method (Figure 4D). This should not be interpreted that the combined method of the present invention provided an inferior result as it should be noted that the digestion of proteins can modify their staining properties. Proteins remaining in the gel were very low in both IFG and combined methods (Figures 5B and 5D).

In a second experiment, these samples were analysed with MALDI-MS for peptide mass fingerprinting detection.

The results of these tests are summarised in Table 1. Recent results based on the OSDT process had previously highlighted the problems pointed out above, relating to partial transblot of basic and high molecular weight proteins. In this case, no identification could

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be obtained for lysozyme (pI 9.2) and pancreatic trypsin
inhibitor (pI 9.3) as well as myosin (MW ≈200 kDa). The
IFG digestion technique was not sufficient to obtain
enough peptides to identify correctly most of the
5    proteins. The combination method of partial IFG followed
by OSDT provided overall the best results in terms of
protein identification. All of the 9 proteins were
identified and that despite the high pI of a lysozyme
(see MALDI-MS spectra in Figs. 6A-6C) and high molecular
10   weight of a myosin (see MALDI-MS spectra in Figs. 7A-7C)
of the analysed proteins. Figures 6A, 7A relate to OSDT,
6A, 7B to IFG digestion and 6C, 7C to the combined method
of the invention. Note in the combined procedure the
greater number of fragments generated and the greater
15   fragmentation of the marker proteins of m.w. 1498.82 and
2095.08 used for internal calibration.

Table 1

| Protein name | pI[1] | MW (kDa)[1] | OSDT | | IFG | | Combined | |
|---|---|---|---|---|---|---|---|---|
| | | | No. Pept[2] | Prot Id[3] | No. Pept[2] | Prot Id[3] | # of Pept[2] | Prot Id[3] |
| Bovine pancreatic trypsin inhibitor | 9.24 | 6.5 | 0 | – | 1 | – | 4 | + |
| Chicken lysozyme | 9.3 | 14.3 | 0 | – | 2 | – | 7 | + |
| Soybean trypsin inhibitor | 4.6 | 20.1 | 7 | + | 1 | – | 7 | + |
| Bovine carbonic anhydrase | 7.9 | 29.0 | 9 | + | 0 | – | 4 | + |
| Chicken ovalbumin | 5.2 | 42.8 | 7 | + | 4 | – | 5 | + |
| Bovine serum albumin | 5.6 | 66.4 | 4 | + | 3 | – | 12 | + |
| Rabbit Phosphorylase b | 6.8 | 97.2 | 12 | + | 1 | – | 17 | + |
| E.coli β-Galactosidase | 5.3 | 118.1 | 20 | + | 7 | + | 19 | + |
| Rabbit myosin[4] | -- | 223 | 0 | – | 2 | – | 10 | + |
| % Identification | -- | -- | -- | 67% | | 11% | | 100% |

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1     pI and molecular weight were calculated with the "Compute pI/Mw" tool (available on the ExPASy server)

2     Number of peptides identified that correspond to correct enzymatic residue-specific fragments of the protein under test (determined with FindMod tool)

3     +/-: Correct/incorrect identification of the protein using the peptide mass fingerprints obtained from MALDI-MS spectra, as determined by PeptIdent (Available on the ExPASy server)

4     Myosin was correctly identified from rabbit skeletal muscle, using the TREMBL database

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Thus, it has been shown that the method of the present invention can identify proteins having a wide range of pI value and molecular weight. First, trypsin digested proteins in the gel without restriction of mass

5   and/or pI. This step resulted in peptides of high average molecular weight corresponding to several missed cleavage, but their lower molecular weights were lower than that of the original protein. For a few peptides, pI and hydrophobicity were also lower than for the entire

10  protein. Secondly, these peptides were easily extracted by electroblotting and digested again during the OSDT procedure. The method gave good mass spectra and subsequent identification for 9 out of 9 analysed proteins.

15  The myosin protein referred to above cannot be immunoblotted by conventional methods, even when using CAPS buffer with 0.01% SDS. However, it can be immunoblotted by the above-described transblotting procedure. By use of a monoclonal antibody, myosin can

20  be detected on the PVDF membrane.

Each of the above-mentioned publications is herein incorporated by reference to the extent to which it is relied on herein.

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CLAIMS

1.    A kit comprising:

a)    a first polypeptide-cleaving reagent suitable for incorporating in an electrophoretic gel;

5    b)    at least one hydrophilic membrane suitable for use in transblotting of polypeptides separated on an electrophoretic gel, the membrane having at least one second polypeptide-cleaving reagent immobilised thereon; and

10    c)    a hydrophobic collection member suitable for receiving thereon fragments of the separated polypeptides transferred thereto by transblotting.

2.    The kit of Claim 1, wherein the hydrophilic membrane and hydrophobic collection member are provided as a pre-

15    formed assembly.

3.    The kit of Claim 1 or 2, wherein the second polypeptide-cleaving reagent is immobilised on the hydrophilic membrane by covalent bonding.

4.    The kit of Claim 3, wherein the second polypeptide-

20    cleaving reagent is immobilised through an amide linkage formed between (1) the functional groups on the hydrophilic membrane selected from the group consisting of activated carbonyl groups, carboxylic acid groups and carboxylic acid derivative groups capable of reacting

25    with an amino group, and (2) an amino group of the polypeptide-cleaving reagent.

5.    The kit of Claim 1, 2, 3 or 4, wherein the polypeptide-cleaving reagents are enzymes, which may be the same or different.

30    6.    The kit of Claim 5, wherein each enzyme comprises a protease.

7.    The kit of Claim 6, wherein the protease comprises trypsin.

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8.   The kit of Claim 1, 2, 3, 4, 5, 6 or 7, further comprising:

d)   a buffer suitable for at least partially rehydrating a gel on which at least one polypeptide has been isolated and which has been dehydrated.

9.   The kit of Claim 1, 2, 3, 4, 5, 6, 7 or 8, wherein the hydrophobic collection member is a self-supporting membrane.

10.  A method of identifying or characterising polypeptides which have been isolated on a gel by electrophoresis, comprising the steps of:

a)   providing a gel on which at least one polypeptide has been isolated;

b)   incorporating a first polypeptide-cleaving reagent in the gel;

c)   providing adjacent to the gel at least one hydrophilic membrane on which is immobilised at least one second polypeptide-cleaving reagent;

d)   providing a hydrophobic collection member suitable for receiving thereon fragments of polypeptide transferred thereto from the gel by transblotting, said hydrophobic layer being positioned beyond the hydrophilic membrane in a direction of movement of the fragments of polypeptide;

e)   transblotting the polypeptides from the full gel, on which the polypeptide or polypeptides were isolated, through the hydrophilic membrane or membranes, under conditions effective to cause them to be cleaved into fragments by the second polypeptide-cleaving reagent, to the hydrophobic layer; and

f)   identifying or characterising the fragments collected on the hydrophobic collection member.

11.  The method of Claim 10, which further comprises:

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g)    identifying or characterising the polypeptide from which the fragments were derived.

12.  The method of Claim 10 or 11, wherein the first polypeptide-cleaving reagent is incorporated in the gel by dehydrating the gel and then at least partially rehydrating it with a buffer containing the polypeptide-cleaving reagent.

13.  The method of Claim 10, 11 or 12, wherein the immobilisation of the second polypeptide-cleaving reagent is by covalent bonding thereof to the hydrophilic membrane.

14.  The method of Claim 10, 11, 12 or 13, wherein both the polypeptide-cleaving reagents are enzymes, which may be the same or different.

15.  The method of Claim 14, wherein either or both enzymes cleave the polypeptide in its main chain.

16.  The method of Claim 14, wherein either or both enzymes cleave the polypeptide in a side-chain thereof.

17.  The method of Claim 14, wherein both enzymes are trypsin and the electroblotting is carried out in a buffer of pH from 8 to 9.

18.  The method of Claim 10, 11, 12, 13, 14, 15, 16 or 17, wherein the voltage at which the electroblotting is carried out is adjusted to provide a slower than normal transfer, so as to extend the residence time of the polypeptide in the proximity of the cleavage reagent.

19.  The method of Claim 10, 11, 12, 13, 14, 15, 16, 17 or 18, wherein the electroblotting is carried out under conditions which provide either (1) a discontinuous current from anode to cathode or (2) an alternating current biased in the anode to cathode direction.

20.  The method of Claim 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19, wherein the fragments are identified by mass spectrometry.

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21. The method of Claim 20, wherein the membrane is scanned directly by matrix-assisted laser desorption/ionisation time of flight spectrometry and the data obtained therefrom compared with a database, using a
5   computer program, to provide automated polypeptide identification.

## Fig.1



- Cathode (-)
- Filter paper
- Cathodeic collection membrane
- SDS PAGE gel
- Hydrophilic membrane with immobilised enzyme
- Anodic collection membrane
- Filter paper
- Anode (+)

## Fig.2



- Cathode (-)
- Filter paper
- SDS PAGE gel
- 1st ⎫ Hydrophilic membranes
- 2nd ⎬ with immobilised enzyme
- Anodic collection membrane
- Filter paper
- Anode (+)

## Fig.3

PROTEIN

Myosin (Rabbit)
β Galactosidase (E. Coli)
Phosphorylase b (Rabbit)
Serum albumin (Bovine)
Ovalbumin (Chicken)
Carbonic anhydrase (Bovine)
Trypsin inhibitor (Soybean)
Lysozyme (Chicken)
Pancreatic trypsin inhibitor (Bovine)



*Fig.4*

D

C

B

A

MW (kDa)

200
116.4
97.2
66.4

42.7

29.0

20.1

14.3

6.5

Fig.5

Fig.6A
IFG digestion

## Fig.6B
### OSDT process

Fig.6C

Combined method
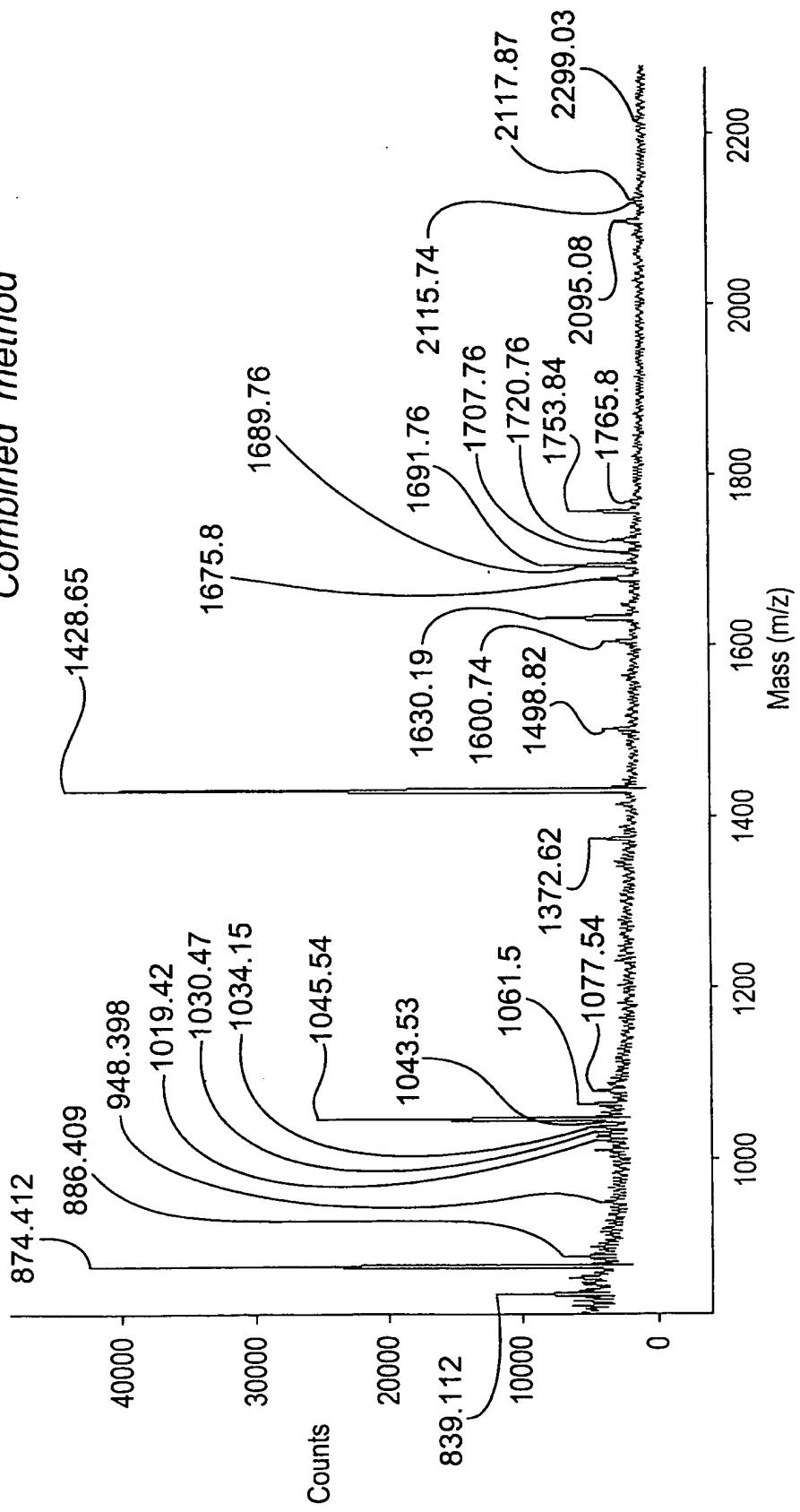
Fig. 7A
IFG digestion
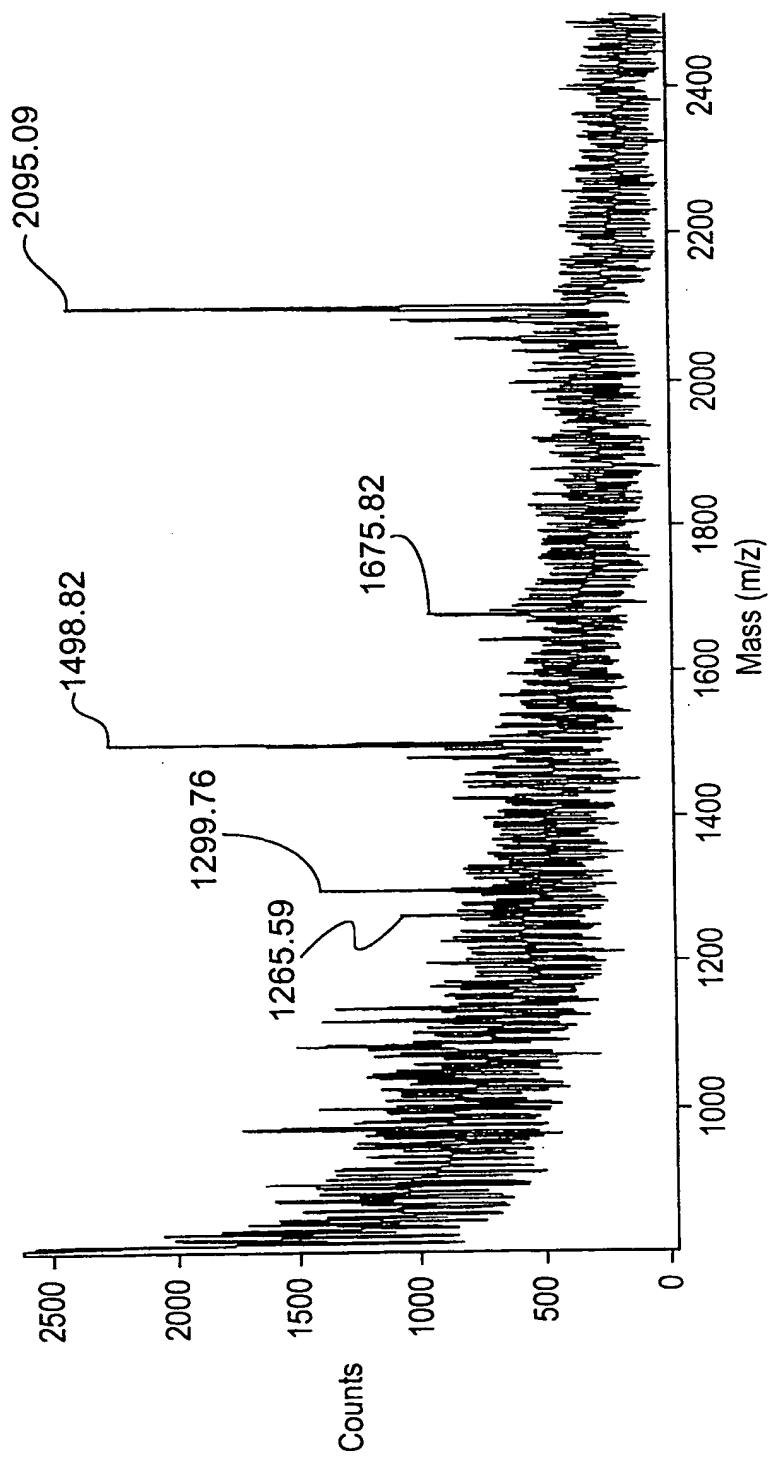
Fig. 7B
OSDT process
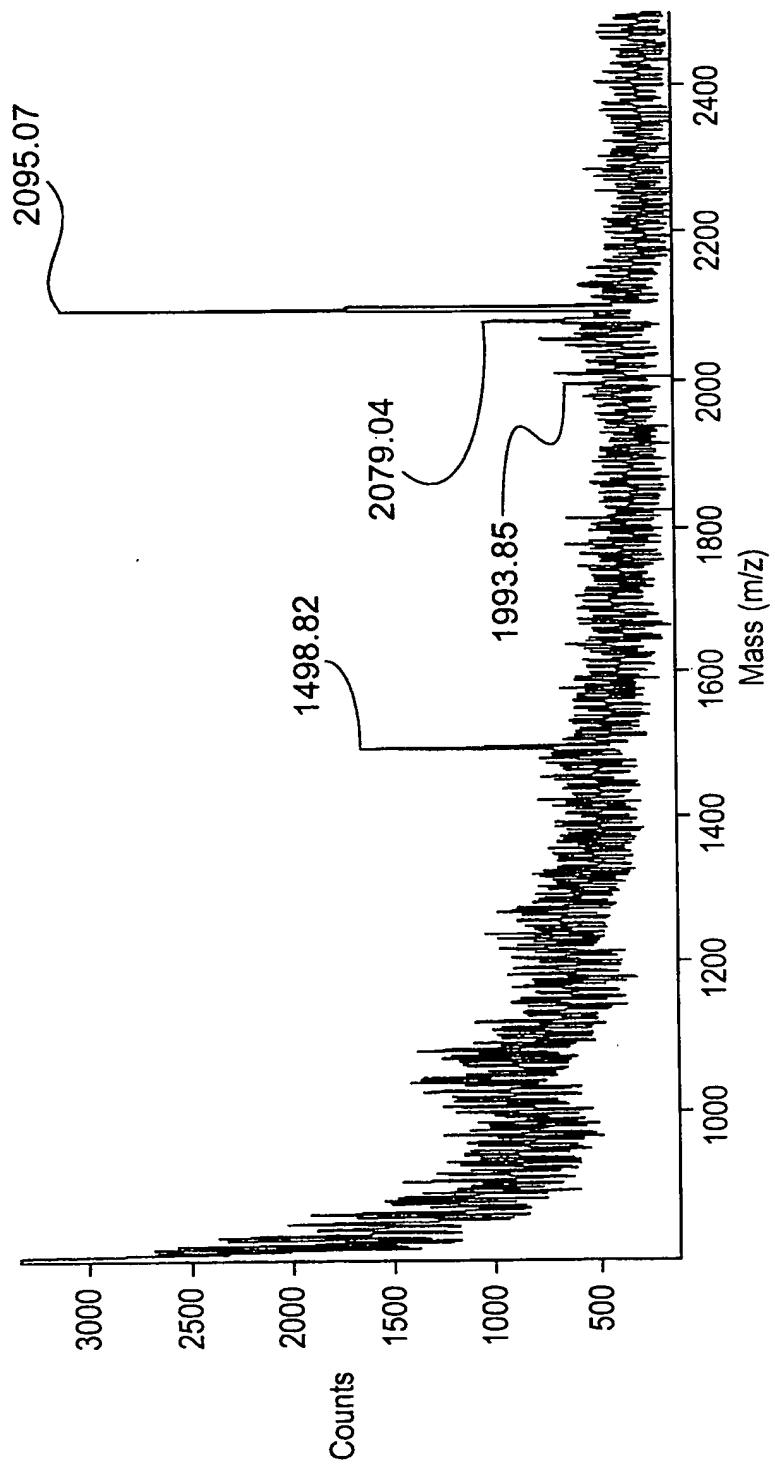
# Fig. 7C
## Combined method
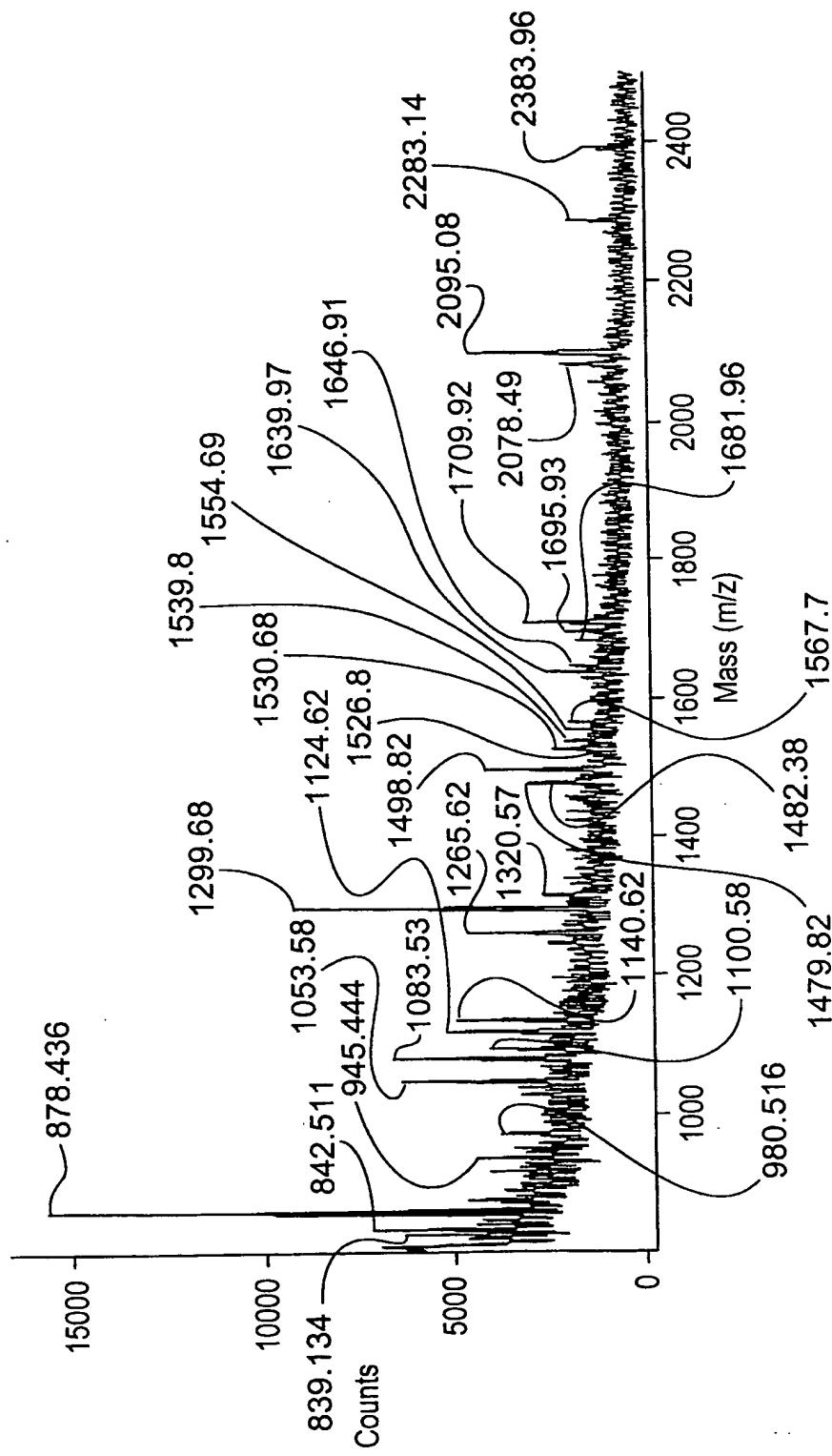
# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER
IPC 7    G01N33/52    G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched  (classification system followed by classification symbols)

IPC 7    G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the  international search (name of data base and,  where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DE 44 08 034 C (BRUKER FRANZEN ANALYTIK GMBH) 13 July 1995 (1995-07-13) abstract column 7, line 68 -column 8, line 5 claims 5-12 | 1-21 |

[ ] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
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| European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Hoekstra, S |

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1

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| | | US | 5595636 A | 21-01-1997 |

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(54) Title: METHOD AND APPARATUS FOR DESORPTION AND IONIZATION OF ANALYTES

(57) Abstract

This invention relates generally to methods and apparatus for desorption and ionization of analytes for the purpose of subsequent scientific analysis by such methods, for example, as mass spectrometry or biosensors. More specifically, this invention relates to the field of mass spectrometry, especially to the type of matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry used to analyze macromolecules, such as proteins or biomolecules. Most specifically, this invention relates to the sample probe geometry, sample probe composition, and sample probe surface chemistries that enable the *selective* capture and desorption of analytes, including intact macromolecules, directly from the probe surface into the gas (vapor) phase without added chemical matrix.

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# METHOD AND APPARATUS FOR
## DESORPTION AND IONIZATION OF ANALYTES

### BACKGROUND OF THE INVENTION

This invention relates generally to methods and apparatus for

5      desorption and ionization of analytes for the purpose of subsequent scientific

analysis by such methods, for example, as mass spectrometry (MS) or

biosensors.   Generally, analysis by mass spectrometry involves the

vaporization and ionization of a small sample of material, using a high energy

source, such as a laser, including a laser beam.   The material is vaporized

10      from the surface of a probe tip into the gas or vapor phase by the laser beam,

and, in the process, some of the  individual molecules are ionized by the gain

of a proton.   The positively charged ionized molecules are then accelerated

through a short high voltage field and let fly (drift) into a high vacuum

chamber, at the far end of which they strike a sensitive detector surface.

15      Since the time-of-flight is a function of the mass of the ionized molecule, the

elapsed time between ionization and impact can be used to determine the

molecule's mass which, in turn, can be used to identify the presence or

absence of known molecules of specific mass.

All known prior art procedures which present proteins or other large

20      biomolecules on a probe tip for laser desorption/ionization time-of-flight mass

spectrometry (TOF) rely on the preparation of a crystalline solid mixture of

the protein or other analyte molecule in a large molar excess of acidic matrix material deposited on the bare surface of a metallic probe tip. (The sample probe tip typically is metallic, either stainless steel, nickel plated material or platinum). Embedding the analyte in such a matrix was thought to be

5      necessary in order to prevent the destruction of analyte molecules by the laser beam. The laser beam strikes the solid mixture on the probe tip and its energy is used to vaporize a small portion of the matrix material along with some of the embedded analyte molecules. Without the matrix, the analyte molecules are easily fragmented by the laser energy, so that the mass, and

10     identity, of the original macromolecule is very difficult or impossible to determine.

       This prior art procedure has several limitations which have prevented its adaptation to automated protein or other macrobiological molecular analysis. First, in a very crude sample it is necessary to partially fractionate

15     (or otherwise purify the sample as much as possible) to eliminate the presence of excessive extraneous materials in the matrix/analyte crystalline or solid mixture. The presence of large quantities of components may depress the ion signal (either desorption, ionization and/or detection) of the targeted analyte. Such purification is time-consuming, expensive, typically results in low

20     recovery (or complete loss) of the analyte, and would be very difficult to do in an automated analyzer.

       Second, while the amount of analyte material needed for analysis by the prior art method is not large (typically in a picomole range), in some circumstances, such as tests on pediatric patients, analyte fluids are available

25     only in extremely small volumes (microliters) and may be needed for

performing several different analyses. Therefore, even the small amount (i.e., volume) needed for preparation of the analyte/matrix crystalline mixture for a single analysis may be significant. Also, only a tiny fraction (a few thousandths or less) of analyte used in preparing the solid analyte/matrix

5      mixture for use on the probe tip is actually consumed in the desorption or mass spectrometric analysis. Any improvement in the prior art procedure which would make it possible to 1) use much less analyte, 2) to locate the analyte or multiple analytes on the probe tip or surface in a predetermined location, 3) to perform repeated analyses of the same aliquot of analyte (e.g.,

10     before and after one or more chemical and or enzymatic reactions), and 4) to conduct the test in a more quantitative manner, would be *highly advantageous* in many clinical areas.

Third, the analyte protein, or other macromolecule, used in preparing the solid solution of analyte/matrix for use on the probe tip is not suitable for

15     any subsequent chemical tests or procedures because it is bound up (i.e., embedded) in the matrix material. Also, all of the matrix material used to date is strongly acidic, so that it would adversely affect many chemical reactions which might be attempted on the mixture in order to modify the analyte molecules for subsequent examination. Any improvement in the

20     procedure which made it possible to conduct subsequent chemical modifications or reactions on the analyte molecules, without removing them from the matrix or the probe tip or without "matrix" altogether, would be of enormous benefit to researchers and clinicians.

The first successful molecular mass measurements of *intact* peptides

25     and small proteins (only up to about 15 kDa) by any form of mass

spectrometry were made by bombarding surfaces with high energy particles (plasma desorption and fast atom bombardment mass spectrometry); this breakthrough came in 1981 and 1982. Improvements came in 1985 and 1986, however, yield (signal intensities), sensitivity, precision, and mass accuracy

5    remained relatively low. Higher molecular mass proteins (about 20 to 25 kDa) were not observed except on rare occasions; proteins representing average molecular weights (approximately 70 kDa) were not ever observed with these methods. Thus, evaluation of most proteins by mass spectrometry remains unrealized.

10   In 1988, Hillenkamp and his coworkers used UV laser desorption time-of-flight mass spectrometry and discovered that when proteins of relatively high molecular mass were deposited on the probe tip in the presence of a very large molar excess of an acidic, UV absorbing chemical matrix (nicotinic acid) they could be desorbed in the intact state. This new technique is called

15   *matrix-assisted* laser desorption/ionization (MALDI) time-of-flight mass spectrometry. Note that laser desorption time-of-flight mass spectrometry (without the chemical matrix) had been around for some time, however, there was little or no success determining the molecular weights of large intact biopolymers such as proteins and nucleic acids because they were fragmented

20   (destroyed) upon desorption. Thus, prior to the introduction of a chemical matrix, laser desorption mass spectrometry was essentially useless for the detection of specific changes in the mass of intact macromolecules. Note that the random formation of matrix crystals and the random inclusion of analyte molecules in the solid solution is prior art.

There are a number of problems and limitations with the prior art methods. For example, previously, it has been found that it is difficult to wash away contaminants present in analyte or matrix. Other problems include formation of analyte-salt ion adducts, less than optimum solubility of

5    analyte in matrix, unknown location and concentration of analyte molecules within the solid matrix, signal (molecular ion) suppression "poisoning" due to simultaneous presence of multiple components, and selective analyte desorption/ionization. Prior investigators, including Karas and Hillenkamp have reported a variety of techniques for analyte detection using mass

10   spectroscopy, but these techniques suffered because of inherent limitations in sensitivity and selectivity of the techniques, specifically including limitations in detection of analytes in low volume, undifferentiated samples. (Hillenkamp, *Bordeaux Mass Spectrometry Conference Report*, pp. 354-62 (1988); Karas and Hillenkamp, *Bordeaux Mass Spectrometry Conference*

15   *Report*, pp. 416-17 (1988); Karas and Hillenkamp, *Analytical Chemistry*, 60:2299 (1988); Karas, et al., *Biomed. Environ. Mass Spectrum* (in press).) The use of laser beams in time-of-flight mass spectrometers is shown, for example, in U.S. Pat. Nos. 4,694,167; 4,686,366, 4,295,046, and 5,045,694, incorporated by reference.

20   The successful volatilization of high molecular weight biopolymers, without fragmentation, has enabled a wide variety of biological macromolecules to be analyzed by mass spectrometry. More importantly perhaps, it has illustrated the potential of using mass spectrometry more creatively to solve problems routinely encountered in biological research.

25   Most recent attention has been focused on the utility of matrix-assisted laser

desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), largely because it is rapid (min), sensitive (< pmol sample required), and permits complex mixtures to be analyzed.

Although MALDI-TOF MS continues to be useful for the static determination/verification of mass for individual analytes, in the case of biopolymers, it is often differences in mass that provide the most important information about unknown structures. Thus, for routine use in structural biology, an unfortunate limitation of the MALDI-TOF MS technique relates to sample preparation and presentation (deposition) on an inert probe element surface, specifically, the requirement that analytes be embedded (i.e., co-solidified) on the probe surface in a freshly prepared matrix of crystalline organic acid. The random distribution of analyte in a heterogeneous display of crystal matrix on the probe element surface requires the deposition of far more analyte or sample than is needed for the laser desorption process, even for the collection of more than adequate mass spectra (e.g., multiple sets of 100 shots each). The remaining portion of the analyte is usually not recovered for additional analyses or subsequent characterizations. Even though 1 to 10 pmol (sometimes less) of analyte are typically required for deposition on the probe surface, it has been estimated that less than a few attomoles are consumed during laser desorption. Thus, only 1 part in $10^5$ or $10^6$ of the applied analyte may be necessary; the rest is lost.

Another important loss of potential data associated with the embedding of analyte in a solid matrix is the reduction or the complete elimination of ability to perform subsequent chemical and/or enzymatic modifications to the embedded analyte (e.g., protein or DNA) remaining on the probe surface.

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Only another aliquot of analyte, or the ability to recover the embedded analyte free of matrix (difficult with low recovery), allows what we now refer to as differential mass spectrometry to be performed to derive structural data.

In addition, there has been limited application of MS in biological
5      fields, likely due to the fact that many biologists and clinicians are intimidated by MS and/or skeptical in regard to its usefulness. Further, MS is perceived as inaccessible or too costly, particularly because SDS polyacrylamide gel electrophoresis is an adequate substitute in some instances where MALDI would be applied (e.g., separation of crude biological fluids).
10     In addition, MALDI has had little exposure in biological and clinical journals.


## SUMMARY OF THE INVENTION

An object of the invention is to provide improved methods, materials composition and apparatus for coupled adsorption, desorption and ionization of multiple or selected analytes into the gas (vapor) phase.

15     Another object is to provide a method and apparatus for affinity-directed detection of analytes, including desorption and ionization of analytes in which the analyte is not dispersed in a matrix solution or crystalline structure but is presented within, on or above an attached surface of energy absorbing "matrix" material through molecular recognition events, in a
20     position where it is accessible and amenable to a wide variety of chemical, physical and biological modification or recognition reactions.

Another object is to provide such a method and apparatus in which the analyte material is chemically bound or physically adhered to a substrate forming a probe tip sample presenting surface.

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A further object is to provide means for the modification of sample presenting surfaces with energy-absorbing molecules to enable the successful desorption of analyte molecules without the addition of exogenous matrix molecules as in prior art.

5          A further object is to provide the appropriate density of energy-absorbing molecules bonded (covalently or noncovalently) in a variety of geometries such that mono layers and multiple layers of attached energy-absorbing molecules are used to facilitate the desorption of analyte molecules of varying masses.

10         A further object is to provide multiple combinations of surfaces modified with energy-absorbing molecules, affinity-directed analyte capture devices, phototubes, etc.

An additional object is to provide such a method and apparatus in which the substrate forming the probe tip or other sample presenting surface

15      is derivatized with one or more affinity reagents (a variety of densities and degrees of amplification) for selective bonding with predetermined analytes or classes of analytes.

A further object is to provide such a system in which the affinity reagent chemically bonds or biologically adheres to the target analyte or class

20      of analytes.

A still further object is to provide a method and apparatus for desorption and ionization of analytes in which unused portion of the analytes contained on the presenting surface remain chemically accessible, so that a series of chemical, enzymatic or physical treatments of the analyte may be

25      conducted, followed by sequential analyses of the modified analyte.

A further object is to provide a method and apparatus for the combined chemical or enzymatic modifications of target analytes for the purpose of elucidating primary, secondary, tertiary, or quaternary structure of the analyte and its components.

5          Another object is to provide a method and apparatus for desorption and ionization of analyte materials in which cations other than protons (H⁺) are utilized for ionization of analyte macromolecules.

Thus, in accomplishing the foregoing objects, there is provided in accordance with the present invention, an apparatus for measuring the mass

10         of an analyte molecule of an analyte sample by means of mass spectrometry, said apparatus comprising a spectrometer tube; a vacuum means for applying a vacuum to the interior of said tube; electrical potential means within the tube for applying an accelerating electrical potential to desorbed analyte molecules from said analyte sample; sample presenting means removably

15         insertable into said spectrometer tube, for presenting said analyte sample in association with surface associated molecule for promoting desorption and ionization of said analyte molecules, wherein said surface molecule is selected from the group consisting of energy absorbing molecule, affinity capture device, photolabile attachment molecule and combination thereof; an analyte

20         sample deposited on said sample presenting means in association with said surface associated molecules, whereby at least a portion of said analyte molecules not consumed in said mass spectrometry analysis will remain accessible for subsequent chemical, biological or physical analytical procedures; laser beam means for producing a laser beam directed to said

25         analyte sample for imparting sufficient energy to desorb and ionize a portion

of said analyte molecules from said analyte sample; and detector means associated with said spectrometer tube for detecting the impact of accelerated ionized analyte molecules thereon.

In addition, in accomplishing the foregoing objects, there is provided

5    in accordance with the present invention, a method in mass spectrometry to measure the mass of an analyte molecule, said method comprising the steps of: derivitizing a sample presenting surface on a probe tip face with an affinity capture device having means for binding with an analyte molecule; exposing said derivitized probe tip face to a source of said analyte molecule

10   so as to bind said analyte molecule thereto; placing the derivitized probe tip with said analyte molecules bound thereto into one end of a time-of-flight mass spectrometer and applying a vacuum and an electric field to form an accelerating potential within the spectrometer; striking at least a portion of the analyte molecules bound to said derivitized probe tip face within the

15   spectrometer with one or more laser pulses in order to desorb ions of said analyte molecules from said tip; detecting the mass of the ions by their time of flight within said mass spectrometer; and displaying such detected mass.

Further, in accomplishing the foregoing objects, there is provided in accordance with the present invention, a method of measuring the mass of

20   analyte molecules by means of laser desorption/ionization, time-of-flight mass spectrometry in which an energy absorbing material is used in conjunction with said analyte molecules for facilitating desorption and ionization of the analyte molecules, wherein the improvement comprises presenting the analyte molecules on or above the surface of the energy absorbing material, wherein

25   at least a portion of the analyte molecules not desorbed in said mass

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spectrometry analysis remain chemically accessible for subsequent analytical procedures.

Additionally, in accomplishing the foregoing objects, there is provided in accordance with the present invention, an apparatus for facilitating

5     desorption and ionization of analyte molecules, said apparatus comprising: a sample presenting surface; and surface associated molecules, wherein said surface associated molecules are selected from the group consisting of energy absorbing molecule, affinity capture device, photolabile attachment molecule and combination thereof, said surface associated molecules associated with

10    said sample presenting surface and having means for binding with said analyte molecules.

Further, there is provided a method for capturing analyte molecules on a sample presenting surface and desorbing/ionizing said captured analyte molecules from said sample presenting surface for subsequent analysis, said

15    method comprising: derivitizing said sample presenting surface with an affinity capture device or photolabile attachment molecule having means for binding with said analyte molecules; exposing said derivitized sample present surface to a sample containing said analyte molecules; capturing said analyte molecules on said derivitized sample presenting surface by means of said

20    affinity capture device or photolabile attachment molecule; and exposing said analyte molecules, while bound to said derivitized sample presenting surface by means of said affinity capture device or photolabile attachment molecule, to an energy or light source to desorb at least a portion of said analyte molecules from said surface.

Additionally, in accordance with the present invention, there is provided a method for preparing a surface for presenting analyte molecules for analysis, said method comprising: providing a substrate on said surface for supporting said analyte; derivitizing said substrate with an affinity capture

5      device or photolabile attachment molecule having means for selectively bonding with said analyte; and a means for detecting said analyte molecules bonded with said affinity capture device or photolabile attachment molecule.

Further, in accomplishing the foregoing objects, there is provided in accordance with the present invention, a sample probe for promoting

10     desorption of intact analytes into the gas phase comprising: a sample presenting surface; and an energy absorbing molecule associated with said sample presenting surface, wherein said sample probe promotes desorption of an intact analyte molecule positioned on, above or between the energy absorbing molecules when said sample probe is impinged by an energy source.

15     Further, the energy absorbing molecule in the probe is selected from the group consisting of cinnamamide, cinnamyl bromide, 2, 5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid.

Additionally, in accomplishing the foregoing objects, there is provided in accordance with the present invention, a sample probe for desorption of

20     intact analyte into the gas phase, comprising:      a sample presentation surface; and a surface associated molecule wherein said surface associated molecule is a photolabile attachment molecule having at least two binding sites, wherein at least one site is bound to the sample presentation surface and at least one site is available to bind an analyte and wherein the analyte

25     binding site is photolabile.

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In addition, in accomplishing the foregoing objects there is provided in accordance with the present invention, a sample probe for promoting desorption of intact analytes into the gas phase comprising: a sample presentation surface; and either

5      a mixture of at least two different molecules selected from the group consisting of an affinity capture device, an energy absorbing molecule and a photolabile attachment molecule associated with said sample presentation surface; wherein when an analyte is associated with said sample probe, said sample probe promotes the transition of the analyte into the gas phase when

10     said sample probe is impinged by an energy source; or at least two different affinity capture devices associated with said sample presentation surface; wherein, when said sample probe is impinged by an energy source, said sample probe promotes the transition of an analyte molecule into the gas phase at different rates depending on the affinity capture device associated

15     with said analyte molecule.

In addition, in accomplishing the foregoing objects there is provided in accordance with the present invention, a sample probe for promoting desorption of intact analyte into the gas phase, comprising: a sample presentation surface; and either a surface associated molecule, wherein said

20     surface associated molecule can function both as an energy absorbing molecule and as an affinity capture device; or a surface associated molecule wherein said surface associated molecule is a photolabile attachment molecule having at least two binding sites, wherein at least one site is bound to the sample presentation surface and at least one site is available to bind an

25     analyte and wherein the analyte binding site is photolabile.

Additionally, there is provided in the present invention, a method in mass spectrometry to measure the mass of an analyte molecule, said method comprising the steps of: derivitizing a sample presenting surface on a probe tip face with a photolabile attachment molecule (PAM), wherein said PAM

5    has at least two binding sites, one binding site binds to the sample presenting surface and at least one binding site is available for binding with an analyte molecule; exposing said derivitized probe tip face to a source of said analyte molecule so as to bind said analyte molecule thereto; placing the derivitized probe tip with said analyte molecules bound thereto into one end of a time-of-

10   flight mass spectrometer and applying a vacuum and an electric field to form an accelerating potential within the spectrometer; striking at least a portion of the analyte molecules bound to said derivitized probe tip face within the spectrometer with one or more laser pulses in order to desorb ions of said analyte molecules from said tip; detecting the mass of the ions by their time

15   of flight within said mass spectrometer; and displaying such detected mass.

In addition, there is provided a method of measuring the mass of analyte molecules by means of laser desorption/ionization, time-of-flight mass spectrometry in which a photolabile attachment molecule (PAM) is used in conjunction with said analyte molecules for facilitating desorption and

20   ionization of the analyte molecules, the improvement comprising: presenting the analyte molecules on or above the surface of the PAM, wherein at least a portion of the analyte molecules not desorbed in said mass spectrometry analysis remain chemically accessible for subsequent analytical procedures.

There is further provided in accordance with the present invention, a

25   sample probe for promoting of differential desorption of intact analyte into

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the gas phase, comprising:　　　a sample presentation surface; and at least two different photolabile attachment molecules associated with said sample presentation surface; wherein, when said sample probe is impinged by an energy source, said sample probe promotes the transition of an analyte

5　molecule into the gas phase at different rates depending on the photolabile attachment molecule associated with said analyte molecule.

Additionally, there is provided in accordance with the present invention, a sample probe for promoting desorption of intact analytes into the gas phase comprising: a sample presenting surface; and a photolabile

10　attachment molecule associated with said sample presenting surface; wherein, when said sample probe is impinged by an energy source, said sample probe promotes the transition of an intact analyte molecule into the gas phase.

Further, there is provided in accordance with the present invention, a method for biopolymer sequence determination comprising the steps of:

15　binding a biopolymer analyte to probe tip containing a sample presenting surface having a surface selected molecule selected from the group consisting of an energy absorbing molecule, an affinity capture device, a photolabile attachment molecule and a combination thereof; desorption of biopolymer analyte in mass spectrometry analysis, wherein at least a portion of said

20　biopolymer is not desorbed from the probe tip; analyzing the results of the desorption modifying the biopolymer analyte still bound to the probe tip; and repeating the desorption, analyzing and modifying steps until the biopolymer is sequenced.

Other and further objects, features and advantages will be apparent and the invention more readily understood from a reading of the following specification and by reference to the accompanying drawings forming a part thereof, wherein the examples of the presently preferred embodiments of the

5    invention are given for the purposes of disclosure.


## BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects and advantages of the invention will be apparent from the following specification and from the accompanying drawings.


10    Figure 1A (upper profile) shows the mass spectrum of the three peptides (human histidine rich glycoprotein metal-binding domains (GHHPH)$_2$G (1206 Da), (GHHPH)$_5$G (2904 Da), and human estrogen receptor dimerization domain (D473-L525) (6168.4 Da)) desorbed in the presence of neutralized energy absorbing molecules (sinapinic acid, pH 6.2). Figure 1B

15    (lower profile) shows the sequential *in situ* metal (Cu)-binding of the peptides in the presence of neutral energy absorbing molecules.


Figure 2A (top profile) shows the mass spectrum of the human casein phosphopeptide (5P, 2488 Da) desorbed in the presence of neutralized energy absorbing molecules (sinapinic acid, pH 6.5). Figure 2B (second from top

20    profile) shows the sequential *in situ* 5 min alkaline phosphatase digestion to remove phosphate groups from the phosphopeptide. Figure 2C (third from top profile) shows the mass spectrum of the phosphopeptide after further *in*

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phosphatase digestion in the presence of acidic energy absorbing molecules (2,5 dihydroxybenzoic acid, pH 2) as described in prior art.

Figure 3 shows a composite mass spectra of the $(GHHPH)_5G$ peptide (2904 Da) before (lower profile) and after (upper profile) *in situ* digestion by

5 carboxypeptidase P in the presence of neutralized energy absorbing molecules (sinapinic acid, pH 6.2).

Figure 4 shows a composite matrix-assisted laser desorption mass spectra of peptide mixtures desorbed from solid glass, polypropylene-coated stainless steel, polystyrene-coated stainless steel and solid nylon probe

10 elements.

SEAC

Figure 5, profile A shows the mass spectrum of sperm activating factor (933 Da) and neurotensin (1655 Da) (and their multiple Na-adducts) in the peptide solution unadsorbed by the IDA-Cu(II) surface. Figure 5, profile B,

15 shows the mass spectrum of angiotensin I (1296.5 Da) plus Na-adduct peaks that were selectively adsorbed on the IDA-Cu(II) surface. Figure 5, profile C, and Figure 6, profile C, show the mass spectrum of the same angiotensin I adsorbed on IDA-Cu(II) after water wash. Figure 6, profile D, shows the sequential *in situ* copper-binding (1 and 2 Cu) by affinity adsorbed

20 angiotensin I. Figure 6, profile E, shows the sequential *in situ* trypsin digestion of the affinity adsorbed angiotensin I.

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Figure 7 shows the mass spectrum of myoglobin (4 to 8 fmole) affinity adsorbed on IDA-Cu(II) surface.

Figure 8 (top profile) shows the mass spectrum of synthetic casein peptide (1934 Da) with multiple phosphorylated forms affinity adsorbed from

5        a crude mixture on TED-Fe(III) surface. After sequential *in situ* alkaline phosphatase digestion, only the original nonphosphorylated form remained (lower profile).

Figure 9, profile A, shows the mass spectrum of total proteins in infant formula. Figure 9, profile B, shows the mass spectrum of phosphopeptides in

10       infant formula affinity adsorbed on TED-Fe(III) surface. Figure 9, profile C, shows the mass spectrum of total proteins in gastric aspirate of preterm infant obtained after feeding the infant formula. Figure 9, profile D, shows the mass spectrum of phosphopeptides in the gastric aspirate affinity adsorbed on TED-Fe(III) surface.

15       Figure 10 A shows the composite mass spectra of human and bovine histidine-rich glycoprotein adsorbed on IDA-Cu(II) surface before and after N-glycanase digestion. The mass shifts represent the removal of carbohydrate from the respective glycoproteins. Figure 10 B shows the composite mass spectra of trypsin digested peptides from the deglycosylated proteins of the

20       two species (top profile for human protein, second from bottom profile for bovine protein) and *in situ* Cu(II)-binding of the trypsin digested peptides of the two species (second from top profile for human protein, bottom profile for

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bovine protein; the numbers 1, 2 indicate the number of copper bound).

Figure 10C shows that one such Cu(II)-binding peptide (bottom profile) has

at least 4 His residues which are specifically modified by diethylpyrocarbonate

to form 4 N-carbethoxy-histidyl adducts (1-4, top profile). Figure 10D shows

5       the partial C-terminal sequence of the major Cu-binding peptide in the bovine

histidine rich glycoprotein.


Figure 11 (bottom profile) shows the mass spectrum of rabbit anti-

human lactoferrin immunoglobulin alone (control) affinity adsorbed on sheep

anti-rabbit IgG paramagnetic surface. The top profile shows the mass

10      spectrum of human lactoferrin and rabbit anti-human lactoferrin

immunoglobulin complex affinity adsorbed on sheep anti-rabbit IgG

paramagnetic surface.


Figure 12 shows the mass spectrum of human lactoferrin affinity

adsorbed from preterm infant urine on a anti-human lactoferrin

15      immunoglobulin nylon surface. Figure 13 shows the equivalent mass

spectrum of whole preterm infant urine containing 1 nmole/ml of lactoferrin.


Figure 14 (lower profile) shows the mass spectrum of pure bovine

histidine rich glycoprotein. The upper profile shows the mass spectrum of

bovine histidine rich glycoprotein and fragments affinity adsorbed from

20      bovine colostrum on anti-bovine histidine rich glycoprotein immunoglobulin

surface.


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Figure 15 shows the composite mass spectra of the peptides of follicle stimulating hormone recognized by the different anti-follicle stimulating hormone antibodies.

5 Figure 16 shows the mass spectrum of human lactoferrin affinity adsorbed on a single bead of single-stranded DNA agarose deposited on a 0.5 mm diameter probe element.

Figure 17 shows the mass spectrum of human lactoferrin affinity adsorbed from preterm infant urine on single-stranded DNA surface

Figure 18A shows the composite mass spectra of the total proteins in 10 human duodenal aspirate (lower profile) and the trypsin affinity adsorbed from the aspirate on a soybean trypsin inhibitor surface (upper profile). Figure 18B shows the mass spectrum of trypsin affinity adsorbed from 1 ul of aspirate on a soybean trypsin inhibitor nylon surface.

Figure 19A shows the mass spectrum of biotinylated insulin affinity 15 adsorbed from human urine on a Streptavidin surface. Figure 19B shows the mass spectrum of biotinylated insulin affinity adsorbed from human plasma on a Streptavidin surface.

Figure 20 (upper profile) shows the mass spectrum of total proteins in human serum. Figure 20 (lower profile) shows the mass spectrum of

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serum albumin affinity adsorbed from human serum on a Cibacron-blue surface.

SEND

Figure 21 shows the molecular structure of surface bound cinnamamide; R represents the surface plus cross-linker.

5

Figure 22 (upper profile) shows the mass spectrum of peptide mixtures desorbed from surface bound cinnamamide. Figure 20B (lower profile) shows the mass spectrum of the same peptide mixtures with free cinnamamide.

Figure 23 shows the molecular structure of surface bound cinnamyl bromide; R represents the surface plus cross-linker.

10

Figure 24 (upper profile) shows the mass spectrum of peptide mixtures desorbed from surface bound cinnamyl bromide. Figure 22B (lower profile) shows the mass spectrum of the same peptide mixtures with free cinnamyl bromide.

Figure 25 shows the molecular structure of surface bound MAP-dihydroxybenzoic acid; R represents the surface plus cross-linker.

15

Figure 26 (upper profile) shows the mass spectrum of peptide mixtures desorbed from surface bound MAP alone. Figure 26 (lower profile) shows the

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mass spectrum of the same peptide mixtures desorbed from surface bound MAP-dihydroxybenzoic acid.

Figure 27A shows the mass spectrum (1,200-50,000 m/z region) of myoglobin desorbed from surface bound α-cyano-4-hydroxycinnamic acid.
5    Figure 25B shows the same mass spectrum in the low mass region (0-1200 m/z).

Figure 28 shows the molecular structure of energy absorbing molecules bound to polyacrylamide or nylon or acrylic surface via glutaraldehyde activation.

10    Figure 29 shows the molecular structure of energy absorbing molecules bound to polyacrylamide or nylon or acrylic surface via divinyl sulfone activation.

Figure 30 shows the molecular structure of energy absorbing molecules bound to polyacrylamide or nylon or acrylic surface via
15    dicyclohexylcarbodiimide activation.

Figure 31 shows the molecular structure of energy absorbing molecules bound to polyacrylamide or nylon or acrylic surface with multiple antigenic peptide via dicyclohexylcarbodiimide activation.

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Figure 32 shows the molecular structure of thiosalicylic acid bound to iminodiacetate (IDA)-Cu(II) surface.

Figure 33 shows the mass spectrum of human estrogen receptor dimerization domain desorbed from thiosalicylic acid-IDA-Cu(II) surface.

5      Figure 34 shows the molecular structure of α-cyano-4-hydroxycinnamic acid bound to DEAE surface.

Figure 35 shows the mass spectrum of human estrogen receptor dimerization domain desorbed from sinapinic acid-DEAE surface. Figure 33B shows the mass spectrum of myoglobin desorbed from α-cyano-4-

10     hydroxycinnamic acid DEAE surface.

Figure 36 shows the molecular structure of α-cyano-4-hydroxycinnamic acid bound to polystyrene surface.

<u>SEPAR</u>

Figure 37 shows the C-terminal sequence analysis of surface

15     immobilized via photolytic bond histidine rich glycoprotein metal binding domain.

DETAILED DESCRIPTION OF THE INVENTION

It will be apparent to one skilled in the art that various substitutions
and modifications may be made to the invention disclosed herein without
departing from the scope and the spirit of the invention.

5        The development of new MS probe element compositions with surfaces
that allow the probe element to actively participate in the capture and
docking of specific analytes has recently defined several new opportunities in
the area now being described as Affinity Mass Spectrometry (AMS). In brief,
several types of new MS probe elements have been designed (Hutchens and

10       Yip, *Rapid Commun Mass Spectrom*, 7: 576-580 (1993)) with Surfaces
Enhanced for Affinity Capture (SEAC). To date, SEAC probe elements have
been used successfully to retrieve and tether different classes of biopolymers,
particularly proteins, by exploiting what is known about protein surface
structures and biospecific molecular recognition.

15       Progress in structural biology continues to be limited by the inability
to obtain biopolymer sequence information at an acceptable rate or level of
sensitivity. By utilizing the methods and apparatus of the present invention,
it has been demonstrated that AMS provides an opportunity to relieve this
limitation. Because the immobilized affinity capture devices on the MS probe

20       element surface (i.e., SEAC) determines the location and affinity (specificity)
of the analyte for the probe surface, the subsequent analytical AMS process
is much more efficient for several reasons. First, the location of analyte on
the probe element surface is predetermined. Thus, the subsequent desorption
is no longer dependent on a random search of the probe surface matrix field

25       with the incident laser beam. Second, analyte detection sensitivity (and

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dynamic range) is increased because molecular ionization suppression effects often observed with complex mixtures are eliminated. Third, the tethered analyte that is not actually consumed by the initial laser-induced desorption process remains available for subsequent analyses. If exogenous matrix was used to promote analyte desorption, it is removed, in most cases, without loss of the tethered analyte. The remaining analyte can then be chemically and/or enzymatically modified directly *in situ* (i.e., while still on the probe element). When analyzed again by MS to determine differences in mass, specific structural details are revealed. The entire process of analysis/modification can be repeated many times to derive structural information while consuming only very small quantities of analyte (sometimes only a few femtomoles or less). The demonstrations of protein structure analysis based on AMS have to date included both N- and C-terminal sequence analyses and verification of several types of sequence-specific posttranslational modifications including phosphorylation and dephosphorylation, glycosylation, cysteine residue reactivity, site-specific chemical modifications (e.g., Histidine residues), and ligand binding.

Beyond biopolymer sequence determinations and the solution of individual biopolymer structures, is the ability to understand the structural determinants of functional supramolecular assemblies. The opportunity to investigate the structural determinants of higher order (e.g., quaternary) structures is also presented by AMS. It has been demonstrated by using the present invention that noncovalent molecular recognition events, some not readily observed by more traditional bioanalytical procedures (often requiring disruption of equilibrium and structure dissociating conditions), are

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investigated directly by the evaluation of molecular associations (i.e., recognition) with macromolecular analytes that have been tethered, directly or indirectly, to the probe element surface.

As used herein, "analyte" refers to any atom and/or molecule; including

5        their complexes and fragment ions. In the case of biological macromolecules, including but not limited to: protein, peptides, DNA, RNA, carbohydrates, steroids, and lipids. Note that most important biomolecules under investigation for their involvement in the structure or regulation of life processes are quite large (typically several thousand times larger than $H_2O$).

10       As used herein, the term "molecular ions" refers to molecules in the charged or ionized state, typically by the addition or loss of one or more protons ($H^+$).

As used herein, the term "molecular fragmentation" or "fragment ions" refers to breakdown products of analyte molecules caused, for example, during

15       laser-induced desorption (especially in the absence of added matrix).

As used herein, the term "solid phase" refers to the condition of being in the solid state, for example, on the probe element surface.

As used herein, "gas" or "vapor phase" refers to molecules in the gaseous state (i.e., *in vacuo* for mass spectrometry).

20       As used herein, the term "analyte desorption/ionization" refers to the transition of analytes from the solid phase to the gas phase as ions. Note that the successful desorption/ionization of large, intact molecular ions by laser desorption is relatively recent (circa 1988)--the big breakthrough was the chance discovery of an appropriate matrix (nicotinic acid).

As used herein, the term "gas phase molecular ions" refers to those ions that enter into the gas phase. Note that large molecular mass ions such as proteins (typical mass = 60,000 to 70,000 times the mass of a single proton) are typically *not* volatile (i.e., they do not normally enter into the gas or vapor

5      phase). However, in the procedure of the present invention, large molecular mass ions such as proteins do enter the gas or vapor phase.

As used herein in the case of MALDI, the term "matrix" refers to any one of several small, acidic, light absorbing chemicals (e.g., nicotinic or sinapinic acid) that is mixed in solution with the analyte in such a manner so

10     that, upon drying on the probe element, the crystalline matrix-embedded analyte molecules are successfully desorbed (by laser irradiation) and ionized from the solid phase (crystals) into the gaseous or vapor phase and accelerated as intact molecular ions. For the MALDI process to be successful, analyte is mixed with a freshly prepared solution of the chemical matrix (e.g.,

15     10,000:1 matrix:analyte) and placed on the inert probe element surface to air dry just before the mass spectrometric analysis. The large fold molar excess of matrix, present at concentrations near saturation, facilitates crystal formation and entrapment of analyte.

As used herein, "energy absorbing molecules (EAM)" refers to any one

20     of several small, light absorbing chemicals that, when presented on the surface of a probe element (as in the case of SEND), facilitate the neat desorption of molecules from the solid phase (i.e., surface) into the gaseous or vapor phase for subsequent acceleration as intact molecular ions. The term EAM is preferred, especially in reference to SEND. Note that analyte

25     desorption by the SEND process is defined as a *surface*-dependent process

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(i.e., *neat* analyte is placed on a surface composed of bound EAM). In contrast, MALDI is presently thought to facilitate analyte desorption by a volcanic eruption-type process that "throws" the entire surface into the gas phase. Furthermore, note that some EAM when used as free chemicals to embed analyte molecules as described for the MALDI process will *not* work (i.e., they do not promote molecular desorption, thus they are not suitable matrix molecules).

As used herein, "probe element" or "sample presenting device" refers to an element having the following properties: it is inert (for example, typically stainless steel) and active (probe elements with *surfaces enhanced* to contain EAM and/or molecular capture devices).

As used herein, "MALDI" refers to Matrix-Assisted Laser Desorption/Ionization

As used herein, "TOF" stands for Time-of-Flight.

As used herein, "MS" refers to Mass Spectrometry.

As used herein "MALDI-TOF MS" refers to Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

As used herein, "ESI" is an abbreviation for Electrospray ionization.

As used herein, "chemical bonds" is used simply as an attempt to distinguish a rational, deliberate, and knowledgeable manipulation of known classes of chemical interactions from the poorly defined kind of general adherence observed when one chemical substance (e.g., matrix) is placed on another substance (e.g., an inert probe element surface). Types of defined chemical bonds include electrostatic or ionic (+/-) bonds (e.g., between a positively and negatively charged groups on a protein surface), covalent bonds

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(very strong or "permanent" bonds resulting from true electron sharing), coordinate covalent bonds (e.g., between electron donor groups in proteins and transition metal ions such as copper or iron), and hydrophobic interactions (such as between two noncharged groups).

5          As used herein, "electron donor groups" refers to the case of biochemistry, where atoms in biomolecules (e.g, N, S, O) "donate" or share electrons with electron poor groups (e.g., Cu ions and other transition metal ions).

The present invention uses a general category of probe elements (i.e.,
10        sample presenting means) with Surfaces Enhanced for Laser Desorption/Ionization (SELDI), within which there are three (3) separate subcategories. Surfaces Enhanced for Neat Desorption (SEND) where the probe element *surfaces* (i.e., sample presenting means) are designed to contain Energy Absorbing Molecules (EAM) instead of "matrix" to facilitate
15        desorption/ionizations of analytes added directly (neat) to the surface. Note that this category 1 (SEND) is used alone or in combination with Surfaces Enhanced for Affinity Capture (SEAC)(category 2), where the probe element surfaces (i.e., sample presenting means) are designed to contain chemically defined and/or biologically defined affinity capture devices to facilitate either
20        the specific or nonspecific attachment or adsorption (so-called docking or tethering) of analytes to the probe surface, by a variety of mechanisms (mostly noncovalent). Note that category 2 (SEAC) is used with added matrix or it is used in combination with category 1 (SEND) without added matrix. Thus, the combination of SEND and SEAC actually represents a distinctive
25        category.

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Category 3 involves Surfaces Enhanced for Photolabile Attachment and

Release (SEPAR) where the probe element surfaces (i.e., sample presenting

means) are designed/modified to contain one or more types of chemically

defined crosslinking molecules to serve as *covalent* docking devices. These

5   Photolabile Attachment Molecules (PAM) are bivalent or multivalent in

character, that is, one side is first reacted so as to permanently attach the

PAM to the probe element surface of the sample presenting means, then the

other reactive side(s) of the PAM is ready to be reacted with the analyte

when the analyte makes contact with the PAM-derivatized probe surface.

10   Such surfaces (i.e., sample presenting means) allow for very strong (i.e.,

stable, covalent) analyte attachment or adsorption (i.e., docking or tethering)

processes that are covalent *but reversible* upon irradiation (i.e., photolabile).

Such surfaces represent platforms for the laser-dependent desorption of

analytes that are to be chemically and/or enzymatically modified *in situ* (i.e.,

15   directly on the probe tip) for the purpose of structure elucidation. Only those

analytes on the probe surface that are actually irradiated (small percentage

of total) is desorbed. The remainder of the tethered analytes remain

covalently bound and is modified without loss due to some inadvertent

uncoupling from the surface. Note that the SEPAR category (category 3) is

20   characterized by analyte attachment processes that are reversible upon

exposure to light. However, the light-dependant reversal of the analyte

surface attachment bond(s) does not necessarily enable analyte desorption

into the gas phase per se. In other words, the molecules responsible for the

photolabile attachment of the analytes to the probe surface are *not* necessarily

25   the same as the Energy Absorbing Molecules (EAM) described for SEND. But

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here is an important exception: The present invention includes some hybrid EAM/PAM chemicals that have dual functionality with respect to SEND and SEPAR. That is, some EAM molecules presently used for SEND can be modified to act as mediators of *both* the SEND and SEPAR processes.

5       Similarly, some hybrid affinity capture/PAM chemicals that have dual functionality with respect to SEAC and SEPAR are provided. The present invention uses some affinity capture devices, particularly those that are biologically defined, that are modified to act as mediators of *both* the SEAC and SEPAR processes.

10      The invention herein presents, a sample presenting means (i.e., probe element surface) with surface-associated (or surface-bound) molecules to promote the attachment (tethering or anchoring) and subsequent detachment of tethered analyte molecules in a light-dependent manner, wherein the said surface molecule(s) are selected from the group consisting of photoactive

15      (photolabile) molecules that participate in the binding (docking, tethering, or crosslinking) of the analyte molecules to the sample presenting means (by covalent attachment mechanisms or otherwise). Further, a sample presenting means (composed of one or more of the suitable probe element materials described in previous claims), wherein analyte(s) are bound to the surface said

20      sample presenting means by one or more photolabile bonds so that incident pulse(s) of light (e.g., from one or more lasers) is used to break the photolabile bond(s) tethering the analyte(s) to the probe element surface in a manner that is consistent with the subsequent desorption of the analyte from the stationary (solid) phase surface of the probe into the gas (vapor)

25      phase is also presented.

The chemical specificity(ies) determining the type and number of said photolabile molecule attachment points between the SEPAR sample presenting means (i.e., probe element surface) and the analyte (e.g., protein) may involve any one or more of a number of different residues or chemical

5      structures in the analyte (e.g., His, Lys, Arg, Tyr, Phe, and Cys residues in the case of proteins and peptides). In other words, in the case of proteins and peptides, the SEPAR sample presenting means may include probe surfaces modified with several different types of photolabile attachment molecules to secure the analyte(s) with a plurality of different types of attachment points.

10     The wavelength of light or light intensity (or incident angle) required to break the photolabile attachment(s) between the analyte and the probe element surface *may be the same or different from* the wavelength of light or light intensity (or incident angle) required to promote the desorption of the analyte from the stationary phase into the gas or vapor phase.

15     The photolabile attachment of the analyte(s) to the probe element surface (i.e., sample presenting means), particularly biopolymers such as peptides, proteins, ribonucleic acid (RNA), deoxyribonucleic acids (DNA), and carbohydrates (CHO), may involve multiple points of attachment between the probe surface and the analyte macromolecule. Once the biopolymer is

20     attached via multiple points of attachment, different points in the backbone of the biopolymer may be deliberately cut or fragmented by chemical and/or enzymatic means so that many of the resulting fragments are now separate and distinct analytes, each one still attached (tethered) to the probe surface by one or more photolabile bonds, to be desorbed into the gas phase in

25     parallel for simultaneous mass analyses with a time-of-flight mass analyzer.

This process enables biopolymer (protein, peptides, RNA, DNA, carbohydrate) sequence determinations to be made.

As used herein "affinity" refers to physical and/or chemical attraction between two molecules. Typically used in nature for purposes of structure or regulation of bioactivity (i.e., information transfer). Usually the affinity of one biomolecule for another is quite specific. Used in the present case to describe principle by which molecular analytes of interest are captured. In the case of SEAC, chemicals or biomolecules with a characteristic affinity for the analyte(s) of interest are tethered (bound) to the surface of the probe element to actively "seek" out and selectively bind the desired analyte.

As used herein, "molecular recognition" refers to the interaction event between two molecules with a natural affinity for one another.

As used herein, "molecular capture" refers to the use of tethered biomolecules to attract and bind (capture) other biomolecules for which a specific affinity relationship exists.

As used herein, "passive adsorption" refers to the act of simply placing the analyte (e.g., with matrix).

As used herein, "active docking" refers to the deliberate capture of analyte molecules on the surface of an active probe element as in the case of SEAC.

As referred to herein "stationary phase" means the same as solid phase. In the present context either the probe element surface itself or one of the "external" particulate SEND or SEAC devices used in conjunction with an inert probe element surface.

As used herein, "active surface area" refers to that area of the surface thought or known to participate in the desired reaction or event (e.g., EAM attachment or affinity capture). The active surface area may be significantly less than the total surface area (due to physical effects such as steric

5      hinderance, some of the total area may not be available or useful).

As used herein, "ligand" refers to a typically relatively small molecule (bait) that binds to a large biomolecule (fish). In the present case, ligands are attached (chemically bound) through a linker arm (fishing line) to the probe element surface. This process allows the biomolecular capture event to be

10     localized on the surface (stationary or solid phase).

As used herein, "affinity reagent" refers to an analyte capture device, *viz.*, the class of molecules (both man made, unnatural, natural and biological) and/or compounds which have the ability of being retained on the presenting surface (by covalent bonding, chemical absorption, etc.) while retaining the

15     ability of recognition and bonding to an analyte.

As used herein, "desorption" refers to the departure of analyte from the surface and/or the entry of the analyte into a gaseous phase.

As used herein, "ionization" refers to the process of creating or retaining on an analyte an electrical charge equal to plus or minus one or

20     more electron units.

As used herein, "adduct" refers to the appearance of an additional mass associated with the analyte and usually caused by the reaction of excess matrix (or matrix break-down products) directly with the analyte.

As used herein, "adsorption" - the chemical bonding (covalent and/or noncovalent) of the energy-absorbing molecules, the affinity reagent (i.e., analyte capture device), and/or the analyte to the probe (presenting surface).

One embodiment of the present invention is an apparatus for

5      measuring the mass of an analyte molecule of an analyte sample by means of mass spectrometry, said apparatus comprising: a spectrometer tube; vacuum means for applying a vacuum to the interior of said tube; electrical potential means within the tube for applying an accelerating electrical potential to desorbed analyte molecules from said analyte sample;

10     sample presenting means removably insertable into said spectrometer tube, for presenting said analyte sample in association with surface associated molecule for promoting desorption and ionization of said analyte molecules, wherein said surface molecule is selected from the group consisting of energy absorbing molecule, affinity capture device, photolabile attachment molecule

15     and combination thereof; an analyte sample deposited on said sample presenting means in association with said surface associated molecules; whereby at least a portion of said analyte molecules not consumed in said mass spectrometry analysis will remain accessible for subsequent chemical, biological or physical analytical procedures; laser beam means for producing

20     a laser beam directed to said analyte sample for imparting sufficient energy to desorb and ionize a portion of said analyte molecules from said analyte sample; and detector means associated with said spectrometer tube for detecting the impact of accelerated ionized analyte molecules thereon.

Another embodiment of the present invention is a method in mass

25     spectrometry to measure the mass of an analyte molecule, said method

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comprising the steps of: derivitizing a sample presenting surface on a probe

tip face with an affinity capture device having means for binding with an

analyte molecule; exposing said derivitized probe tip face to a source of said

analyte molecule so as to bind said analyte molecule thereto; placing the

5      derivitized probe tip with said analyte molecules bound thereto into one end

of a time-of-flight mass spectrometer and applying a vacuum and an electric

field to form an accelerating potential within the spectrometer; striking at

least a portion of the analyte molecules bound to said derivitized probe tip

face within the spectrometer with one or more laser pulses in order to desorb

10     ions of said analyte molecules from said tip; detecting the mass of the ions by

their time of flight within said mass spectrometer; and displaying such

detected mass. In an preferred embodiment, this method further comprises

applying a desorption/ionization assisting matrix material to said probe tip

face in association with said affinity capture device. In a more preferred

15     embodiment, the method according further comprises removing said probe tip

from said mass spectrometer; performing a chemical or biological procedure

on said portion of said analyte molecules not desorbed to alter the

composition of said portion of said analyte molecules not desorbed;

reinserting said probe tip with said altered analyte molecules thereon; and

20     performing subsequent mass spectrometry analysis to determine the

molecular weight of said altered analyte molecules.

In an additional embodiment, said affinity capture device is chemically

bonded to said face of said probe tip, physically adhered to said face of said

probe tip, adapted to chemically bond to said analyte molecules, or adapted

25     to biologically adhere to said analyte molecules. In a further embodiment,

said analyte molecules are biomolecules and said affinity reagent is adapted to selectively isolate said biomolecules from an undifferentiated biological sample. In a preferred embodiment, said matrix materials are in the weakly acidic to strongly basic pH range. In a more preferred embodiment, said

5    matrix materials have a pH above 6.0. Further, an additional embodiment presents the face of said probe tip formed of an electrically insulating material.

An additional embodiment of the present invention is a method of measuring the mass of analyte molecules by means of laser

10   desorption/ionization, time-of-flight mass spectrometry in which an energy absorbing material is used in conjunction with said analyte molecules for facilitating desorption and ionization of the analyte molecules, wherein the improvement comprises presenting the analyte molecules on or above the surface of the energy absorbing material, wherein at least a portion of the

15   analyte molecules not desorbed in said mass spectrometry analysis remain chemically accessible for subsequent analytical procedures.

A further embodiment of the present invention is an apparatus for facilitating desorption and ionization of analyte molecules, said apparatus comprising: a sample presenting surface; and surface associated molecules,

20   wherein said surface associated molecules are selected from the group consisting of energy absorbing molecule, affinity capture device, photolabile attachment molecule and combination thereof, said surface associated molecules associated with said sample presenting surface and having means for binding with said analyte molecules.

In a preferred embodiment, said sample presenting surface comprises the surface of a probe tip for use in a time-of-flight mass spectrometry analyzer. In addition, the preferred embodiment presents an affinity capture device or photolabile attachment molecule that is chemically bonded to said

5      sample presenting surface, physically adhered to said sample presenting surface, chemically bonded to said analyte molecules, or is adapted to biologically adhere to said analyte molecules. Further, the preferred embodiment presents analyte molecules are biomolecules and said affinity capture device or photolabile attachment molecule is adapted to selectively

10     isolate said biomolecules from an undifferentiated biological sample.

In addition, the apparatus may have a matrix material deposited on said sample presenting surface in association with said affinity capture device or photolabile attachment molecule. In a more preferred embodiment, the matrix material is in the weakly acidic to strongly basic pH range. In a most

15     preferred embodiment, the matrix material has a pH above 6.0. Additionally, a preferred embodiment includes a sample presenting surface formed of an electrically insulating material.

In an additional embodiment of the present invention, there is presented a method for capturing analyte molecules on a sample presenting

20     surface and desorbing/ionizing said captured analyte molecules from said sample presenting surface for subsequent analysis, said method comprising: derivitizing said sample presenting surface with an affinity capture device or photolabile attachment molecule having means for binding with said analyte molecules; exposing said derivitized sample present surface to a sample

25     containing said analyte molecules; capturing said analyte molecules on said

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derivitized sample presenting surface by means of said affinity capture device

or photolabile attachment molecule; and exposing said analyte molecules,

while bound to said derivitized sample presenting surface by means of said

affinity capture device or photolabile attachment molecule, to an energy or

5      light source to desorb at least a portion of said analyte molecules from said

surface.

A further embodiment of the present invention is a method for

preparing a surface for presenting analyte molecules for analysis, said method

comprising: providing a substrate on said surface for supporting said analyte;

10     derivitizing said substrate with an affinity capture device or photolabile

attachment molecule having means for selectively bonding with said analyte;

and a means for detecting said analyte molecules bonded with said affinity

capture device or photolabile attachment molecule.   In a preferred

embodiment, there is provided the additional step of applying a detection

15     material to said surface.  In a more preferred embodiment, such detection

material comprises a fluorescing species, an enzymatic species, a radioactive

species, or a light-emitting species.

In an additional preferred embodiment, the step of depositing a

desorption/ionization assisting material to said sample presenting surface in

20     association with said affinity capture device or photolabile attachment

molecule is included. In a further preferred embodiment, the energy source

comprises a laser.  In another preferred embodiment, an affinity capture

device is used and said energy source comprises an ion source. Further, a

preferred embodiment may include a portion of said analyte molecules

25     remaining bound to said sample presenting surface after exposure to said

energy source. In a more preferred embodiment, the additional steps of converting at least a portion of the analyte molecules remaining bound on said derivitized sample presenting surface to modified analyte molecules by a chemical, biological or physical reaction, wherein said analyte molecules

5    remain bound to said derivitized sample presenting surface by means of said affinity capture device or photolabile attachment molecule; and exposing said modified analyte molecules to an energy source so as to desorb at least a portion of said modified analyte molecules from said surface are included.

In an embodiment of the present invention, a sample probe for

10   promoting desorption of intact analytes into the gas phase comprising: a sample presenting surface; and an energy absorbing molecule associated with said sample presenting surface, wherein said sample probe promotes desorption of an intact analyte molecule positioned on, above or between the energy absorbing molecules when said sample probe is impinged by an energy

15   source is provided. In a more preferred embodiment, the energy absorbing molecule is selected from the group consisting of cinnamamide, cinnamyl bromide, 2, 5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid. Also in a preferred embodiment, one may utilize a sample presenting surface selected from the group consisting of glass, ceramics, teflon coated magnetic

20   materials; organic polymers and native biopolymers.

In another embodiment of the present invention, there is provided a sample probe for promoting desorption of intact analytes into the gas phase comprising: a sample presenting surface; and        an affinity capture device associated with said sample presenting surface; wherein, when said sample

25   probe is impinged by an energy source, said sample probe promotes the

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transition of an intact analyte molecule into the gas phase. In a preferred

embodiment, the affinity capture device is selected from the group consisting

of metal ions, proteins, peptides, immunoglobulins, nucleic acids,

carbohydrates, lectins, dyes, reducing agents and combination thereof. In

5      another preferred embodiment, the sample presenting surface is selected from

the group consisting of glass, ceramics, teflon coated magnetic materials;

organic polymers and native biopolymers.

An additional embodiment presents a sample probe for desorption of

intact analyte into the gas phase, comprising: a sample presentation surface;

10     and a surface associated molecule wherein said surface associated molecule

is a photolabile attachment molecule having at least two binding sites,

wherein at least one site is bound to the sample presentation surface and at

least one site is available to bind an analyte and wherein the analyte binding

site is photolabile.

15     In another embodiment, there is provided a sample probe for

promoting desorption of intact analytes into the gas phase comprising: a

sample presentation surface; and either a mixture of at least two different

molecules selected from the group consisting of an affinity capture device, an

energy absorbing molecule and a photolabile attachment molecule associated

20     with said sample presentation surface; wherein when an analyte is associated

with said sample probe, said sample probe promotes the transition of the

analyte into the gas phase when said sample probe is impinged by an energy

source; or at least two different affinity capture devices associated with said

sample presentation surface; wherein, when said sample probe is impinged by

25     an energy source, said sample probe promotes the transition of an analyte

molecule into the gas phase at different rates depending on the affinity

capture device associated with said analyte molecule.

In a preferred embodiment, the analyte is selectively desorbed from the

mixture after impingement by the energy source. In another preferred

5        embodiment, the affinity devices are arranged in predetermined arrays. In

a more preferred embodiment, the arrays selectively absorb a plurality of

different analytes.

In a more preferred embodiment, an apparatus of the present invention

is used to quantitate an analyte, wherein the position and quantity of affinity

10       capture devices determines the quantity of analyte absorbed. In another

preferred embodiment, the binding may be selective or non-selective.

In an additional embodiment, a sample probe for promoting desorption

of intact analyte into the gas phase, comprising: a sample presentation

surface; and either a surface associated molecule, wherein said surface

15       associated molecule can function both as an energy absorbing molecule and

as an affinity capture device; or a surface associated molecule wherein said

surface associated molecule is a photolabile attachment molecule having at

least two binding sites, wherein at least one site is bound to the sample

presentation surface and at least one site is available to bind an analyte and

20       wherein the analyte binding site is photolabile.

A different embodiment of the present invention includes a method in

mass spectrometry to measure the mass of an analyte molecule, said method

comprising the steps of: derivitizing a sample presenting surface on a probe

tip face with a photolabile attachment molecule (PAM), wherein said PAM

25       has at least two binding sites, one binding site binds to the sample presenting

surface and at least one binding site is available for binding with an analyte molecule; exposing said derivitized probe tip face to a source of said analyte molecule so as to bind said analyte molecule thereto; placing the derivitized probe tip with said analyte molecules bound thereto into one end of a time-of-

5      flight mass spectrometer and applying a vacuum and an electric field to form an accelerating potential within the spectrometer; striking at least a portion of the analyte molecules bound to said derivitized probe tip face within the spectrometer with one or more laser pulses in order to desorb ions of said analyte molecules from said tip; detecting the mass of the ions by their time

10     of flight within said mass spectrometer; and displaying such detected mass. In a preferred embodiment, the step of applying a desorption/ionization assisting matrix material to said probe tip face in association with said PAM is included. In a more preferred embodiment, an additional steps of removing said probe tip from said mass spectrometer; performing a chemical, biological

15     or physical procedure on said portion of said analyte molecules not desorbed to alter the composition of said portion of said analyte molecules not desorbed; reinserting said probe tip with said altered analyte molecules thereon; and

performing subsequent mass spectrometry analysis to determine the

20     molecular weight of said altered analyte molecules are included. A preferred embodiment may also include PAM being chemically bonded to said face of said probe tip; PAM being chemically bonded to said analyte molecule, wherein said bond between the PAM and the analyte molecule is broken and the analyte molecule is released in a light dependent manner; or, where said

25     analyte molecules are biomolecules, said PAM is adapted to selectively isolate

said biomolecules from an undifferentiated biological sample. In another preferred embodiment, said matrix materials are in the weakly acidic to strongly basic pH range. In a more preferred embodiment, said matrix materials have a pH above 6.0. A preferred embodiment may also include the

5      face of said probe tip being formed of an electrically insulating material.

A further embodiment presents a method of measuring the mass of analyte molecules by means of laser desorption/ionization, time-of-flight mass spectrometry in which a photolabile attachment molecule (PAM) is used in conjunction with said analyte molecules for facilitating desorption and

10     ionization of the analyte molecules, the improvement comprising: presenting the analyte molecules on or above the surface of the PAM, wherein at least a portion of the analyte molecules not desorbed in said mass spectrometry analysis remain chemically accessible for subsequent analytical procedures.

Another embodiment of the present invention is a sample probe for

15     promoting of differential desorption of intact analyte into the gas phase, comprising: a sample presentation surface; and at least two different photolabile attachment molecules associated with said sample presentation surface; wherein, when said sample probe is impinged by an energy source, said sample probe promotes the transition of an analyte molecule into the gas

20     phase at different rates depending on the photolabile attachment molecule associated with said analyte molecule. In a preferred embodiment, the photolabile attachment molecules are arranged in predetermined arrays. In a more preferred embodiment, the arrays selectively absorb a plurality of different analytes.

An additional embodiment of the present invention includes a sample probe for promoting desorption of intact analytes into the gas phase comprising: a sample presenting surface; and a photolabile attachment molecule associated with said sample presenting surface; wherein, when said sample probe is impinged by an energy source, said sample probe promotes the transition of an intact analyte molecule into the gas phase. In a preferred embodiment, and analyte is quantitated, wherein the position and quantity of photolabile attachment molecule determines the quantity of analyte absorbed.

Another embodiment shows a method for biopolymer sequence determination comprising the steps of: binding a biopolymer analyte to probe tip containing a sample presenting surface having a surface selected molecule selected from the group consisting of an energy absorbing molecule, an affinity capture device, a photolabile attachment molecule and a combination thereof; desorption of biopolymer analyte in mass spectrometry analysis, wherein at least a portion of said biopolymer is not desorbed from the probe tip; analyzing the results of the desorption modifying the biopolymer analyte still bound to the probe tip; and repeating the desorption, analyzing and modifying steps until the biopolymer is sequenced. A preferred embodiment presents the biopolymer selected from the group consisting of protein, RNA, DNA and carbohydrate.

The following specific examples describe specific embodiments of the present invention and its materials and methods, are illustrative of the invention and are not intended to limit the scope of the invention.

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The examples of the present invention utilize a time-of-flight mass spectrometer with a high energy source, such as a laser beam, to vaporize the analyte from the surface of a probe tip. In the process, some of the molecules are ionized. The positively charged molecules are then accelerated through

5       a short high voltage field and enter into a field-free flight tube. A sensitive detector positioned at the end of the flight tube gives a signal as each molecular ion strikes it. One skilled in the art recognizes that other modes of detection and ionization can also be used.

## EXAMPLE 1

10      Energy Absorbing Molecules in Aqueous, Neutralized Form

Prior art matrix material used in matrix-assisted laser desorption time-of-flight mass spectrometry are strongly acidic. One of the present discoveries is that analytes is desorbed when mixed with neutralized energy absorbing molecules dissolved in entirely aqueous solvents. By suitable

15      neutralization to pH 6.0 or above, the matrix material is made largely passive to subsequent chemical or enzymatic reactions carried out on the analyte molecules presented on the probe tip surfaces. Since only a small fraction of the analyte molecules are used in each desorption/mass spectrometer measurement, the samples on the probe tips are available for *in situ*

20      sequential chemical or enzymatic modifications. After modification the samples are analyzed by mass spectrometry. Analysis on the same probe tips provides a more accurate determination of the molecule and its characteristics, including its structure.

Mass spectrometry is performed on a modified Vestec model VT2000 or a MAS model SELDI Research Linear time-of-flight mass spectrometer which uses a frequency-tripled output from a Q-switched neodymiumyttrium aluminum garnet (Nd-YAG) pulsed laser (355 nm, 5 ns pulse). Ions desorbed

5    by pulsed laser irradiation are accelerated to an energy of 30 keV and allowed to drift along a 2-meter field free drift region (maintained at $10^{-8}$ torr). Ion signals detected using a 20-stage discrete dynode electron multiplier are amplified by a factor of 10 using a fast preamplifier prior to being recorded using a 200 MS/s transient recorder (LeCroy TR8828D, 8-bit y-axis

10    resolution) or a Tektronix digitizer capable of fast signal averaging. The laster irradiance is adjusted real-time, while monitoring the process on an oscilloscope (Tektronix), in order to achieve optimum ion signal. Data reduction (peak centroid calculations and time to mass/charge conversions) are performed with PC-based software. A VG TOFSpec mass spectrometer

15    which uses a nitrogen laser generating pulsed laser at 335 nm. or a Linear LDI 1700 mass spectrometer which uses a nitrogen laser generating pulsed laser 335 nm. may also be used.

I. Specific Analysis

1. Sinapinic acid (Aldrich Chemical Co., Inc., Milwaukee, WI) is suspended

20    in water at 20 mg/ml (pH 3.88) and neutralized with triethylamine (Pierce, Rockford, IL) to pH 6.2-6.5. An aqueous mixture (1 μl) of synthetic peptides, containing human histidine rich glycoprotein metal-binding domains (GHHPH)₂G (1206 Da), (GHHPH)₅G (2904 Da), and human estrogen receptor dimerization domain (D473-L525) (6168.4 Da) is mixed with 2 μl sinapinic

acid (20 mg/ml water, pH 6.2) on a probe tip and analyzed by laser desorption time-of-flight mass spectrometry. After acquiring five spectra (average 100 laser shots per spectrum), the probe is retrieved, 2 μl of 20 mM Cu(SO)$_4$ is added and the sample is reanalyzed by mass spectrometry. Figure 1A (upper

5     profile) shows the mass spectrum of the three peptides desorbed in the presence of neutralized energy absorbing molecules. Figure 1B (lower profile) shows the *in situ* metal-binding of the peptides in the presence of neutral energy absorbing molecules. The (GHHPH)$_2$G peptide can bind at least 4 Cu(II) , the (GHHPH)$_3$G peptide can bind at least 5 Cu(II) and the

10     dimerization domain can bind at least 1 Cu(II) under the present experimental conditions. Similar result is obtained with α-cyano-4-hydroxycinnamic acid (20 mg/ml water) neutralized to pH 6.5.

2.     An aliquot of 1 μl of human β casein phosphopeptide (R1-K18 + 5P) (2488 Da) is mixed with 1 μl of sinapinic acid (20 mg/ml water) neutralized

15     to pH 6.5, and analyzed by laser desorption time-of-flight mass spectrometry. After acquiring five spectra (average 100 laser shots per spectrum), the probe is removed, the remaining phosphopeptide mixed with the neutralized sinapinic acid is digested directly on the probe tip by 0.5 μl of alkaline phosphatase (Sigma) and incubated at 23°C for 5 min. After acquiring five

20     spectra (average 100 laser shots per spectrum), the probe is removed, further digestion on remaining phosphopeptides is carried out by adding another aliquot of 0.5 μl of alkaline phosphatase and incubated at 23°C for 5 min. The sample is re-analyzed by laser desorption mass spectrometry. Figure 2A (top profile) shows the mass spectrum of the phosphopeptide desorbed in the

presence of neutralized energy absorbing molecules. Figure 2B (second from top profile) shows the *in situ* 5 min alkaline phosphatase digestion to remove phosphate groups from the phosphopeptide. The 0P, 1P and 3P peaks represent the products after removal of five, four and two phosphate groups

5 respectively from the phosphopeptide. Figure 2C (third from top profile) shows that further *in situ* digestion with alkaline phosphatase can result in almost complete removal of all phosphate groups from the phosphopeptide. In contrast, Figure 2D (bottom profile) shows that in the control experiment where *in situ* alkaline phosphatase (0.5 μl) digestion is carried out in the

10 presence of energy absorbing molecules without prior neutralization (e.g. sinapinic acid at pH 3.88 or dihydroxybenzoic acid at pH 2.07), very limited digestion occurred in 10 min at 23°C.


3. An aliquot of 1 μl of (GHHPH)₅G peptide (2904 Da) is mixed with 2 μl of sinapinic acid (20 mg/ml water) neutralized to pH 6.2, and analyzed by laser

15 desorption time-of-flight mass spectrometry. After acquiring five spectra (average 100 laser shotsper spectrum), the remaining peptides mixed with neutralized sinapinic acid are digested directly on the probe tip by 1 μl of carboxypeptidase P (Boehringer Mannheim Corp, Indianapolis, IN) and incubated at 23°C for 30 min. The sample is analyzed by mass spectrometry.

20 Figure 3 shows a composite mass spectra of the peptide before (lower profile) and after (upper profile) *in situ* digestion by carboxypeptidase P in the presence of neutralized energy absorbing molecules. The decrease in mass represents the removal of a Gly residue from the C-terminal of the peptide.

These examples illustrate that neutralized energy absorbing molecules in aqueous solutions are more biocompatible in preserving the structure and function of the analytes even when added in large molar excess. Their presence results in no interference to *in situ* sequential chemical or enzymatic

5        reactions on the remaining analyte.


## EXAMPLE 2

### Nonmetallic Probe Elements (Sample Presenting Surfaces)

It has been found that the probe elements (probe tips or sample presenting surfaces) used in the process of the invention need not be metal

10       or metal-coated, as described in prior art procedures. The sample presenting surfaces are composed of a variety of materials, including porous or nonporous materials, with the porous materials providing sponge-like, polymeric, high surface areas for optimized adsorption and presentation of analyte.

15        Polypropylene or polystyrene or polyethylene or polycarbonate are melted in an open flame and deposited as a thin layer on a 2 mm diameter stainless steel probe element so as to cover it completely. Solid glass rod or solid nylon filaments (up to 1.5 mm diameter) or polyacrylamide rod are cut into 1 cm segments and inserted into the stainless steel probe support.

20       Magnetic stir bars (1.5 x 8 mm, teflon-coated) are inserted into stainless steel probe tip support. An aliquot of 1 µl of peptide mixture containing (GHHPH)$_5$G and human estrogen receptor dimerization domain, is mixed with 2 µl of dihydroxybenzoic acid (dissolved in 30% methanol, 0.1% trifluoroacetic acid) on each of such probe elements and analyzed by laser

desorption time-of-flight mass spectrometry. Figure 4 shows that analytes could be desorbed from several examples of insulating, biocompatible surfaces.

These surfaces can be derivatized (at varying densities) to bind by chemical bonds (covalent or noncovalent) affinity adsorption reagents (affinity

5     capture devices), energy absorbing molecules (bound "matrix"molecules) or photolabile attachment molecules. The geometry of the sample presenting surface is varied (i.e., size, texture, flexibility, thickness, etc.) to suit the need (e.g., insertion into a living organism through spaces of predetermined sizes) of the experiment (assay).


10                              **EXAMPLE 3**

**Affinity-directed laser desorption**

**(Surface Enhanced Affinity Capture, SEAC)**

This example describes the use of existing and new solid phase affinity reagents designed for the (1) capture (adsorption) of one or more analytes, (2)

15     the preparation of these captured analytes (e.g., washing with water or other buffered or nonbuffered solutions to remove contaminants such as salts, and multiple cycles of washing, such as with polar organic solvent, detergent-dissolving solvent, dilute acid, dilute base or urea), and (3) most importantly, the direct transfer of these captured and prepared analytes to the probe

20     surface for subsequent analyte desorption (for detection, quantification and/or mass analysis). Affinity capture devices are immobilized on a variety of materials, including electrically insulating materials (porous and nonporous), flexible or nonrigid materials, optically transparent materials (e.g., glass, including glass of varying densities, thicknesses, colors and with varying

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refractive indices), as well as less reactive, more biocompatible materials (e.g., biopolymers such as agarose, dextran, cellulose, starches, peptides, and fragments of proteins and of nucleic acids). The preferred probe tip, or sample surface, for selective adsorption/presentation of sample for mass

5    analysis are (1) stainless steel (or other metal) with a synthetic polymer coating (e.g., cross-linked dextran or agarose, nylon, polyethylene, polystyrene) suitable for covalent attachment of specific biomolecules or other nonbiological affinity reagents, (2) glass or ceramic, and/or (3) plastics (synthetic polymer). The chemical structures involved in the selective

10   immobilization of affinity reagents to these probe surfaces will encompass the known variety of oxygen-dependent, carbon-dependent, sulfur-dependent, and/or nitrogen-dependent means of covalent or noncovalent immobilization.

I. Surface immobilized metal ion as the affinity capture device

1. Cu(II) ion is chelated by iminodiacetate (IDA) group covalently attached

15   to either porous agarose beads (Chelating Sepharose Fast Flow, Pharmacia Biotech Inc., Piscataway, NJ, ligand density 22-30 μmole/ml gel) or solid silica gel beads (Chelating TSK-SW, ToyoSoda, Japan, ligand density 15-20 μmole/ml gel). A mixture of synthetic peptides containing neurotensin (1655 Da), sperm activating peptide (933 Da) and angiotensin I (1296.5 Da), is

20   mixed with 50 μl packed volume of TSK-SW IDA-Cu(II) at pH 7.0 (20 mM sodium phosphate, 0.5 M sodium chloride) at 23°C for 10 min. The gel is separated from the remaining peptide solution by centrifugation and is then washed with 200 μl sodium phosphate, sodium chloride buffer, pH 7.0 three times to remove nonspecifically bound peptides. Finally, the gel is suspended

25   in 50 μl of water. Aliquots of 2 μl gel suspension and nonadsorbed peptide

solution are mixed with 1 μl of sinapinic acid (dissolved in methanol) on a

stainless steel probe tip and analyzed by laser desorption time-of-flight mass

spectrometry. After acquiring five spectra (average of 100 laser shots per

spectrum) on various spots of the probe tip, the sinapinic acid is removed by

5      methanol. An aliquot of 2 μl of 20 mM $CuSO_4$ is added, then mixed with 1

μl of sinapinic acid and reanalyzed by laser desorption time-of-flight mass

spectrometry. After acquiring another five spectra (average of 100 laser shots

per spectrum) on various spots of the probe tip, the sinapinic acid is removed

by methanol. The remaining peptide adsorbed on IDA-Cu(II) gel beads is

10     then digested with 1 μl of trypsin (Sigma) in 0.1 M sodium bicarbonate, pH

8.0 at 23°C for 10 min in a moist chamber. The gel beads are then washed

with water to remove enzyme and salt before 1 μl of sinapinic acid is added

and the sample analyzed by laser desorption time-of-flight mass spectrometry.

Figure 5A shows the molecular ions (and multiple Na-adducts) of sperm

15     activating factor (933 Da) and neurotensin (1655 Da) in the remaining

peptide solution unabsorbed by the IDA-Cu(II). There is no significant peak

corresponding to angiotensin I (1296.5 Da). The mass spectrum in Figure 5B

shows the angiotensin I plus Na-adduct peaks that are selectively adsorbed

on the IDA-Cu(II) gel. When the IDA-Cu(II) gel is further washed with 500

20     μl of water two times, the resulting mass spectrum shows only the parent

angiotensin I ion and no other adduct peaks (Figures 5 and 6, profiles C).

Figure 6D shows the *in situ* copper binding (1 and 2 Cu) by the angiotensin

peptide. Figure 6E shows the *in situ* trypsin digestion of the angiotensin

peptide at the single Arg2 position in the sequence.

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This example illustrates that: a) laser desorption is successfully carried out on analyte affinity adsorbed on surface-immobilized metal ion; b) once bound, the surface is washed with various solvents to remove all contaminating compounds in the sample to give a very clean mass spectrum

5    of the analyte; c) the affinity capture device selects only the analyte of defined structure (in this case angiotensin I is selectively adsorbed from the peptide mixture by IDA-Cu(II) because this peptide has a free N-terminal and two histidine amino acid residues in the sequence, both properties are required for strong Cu(II)-binding; whereas both sperm activating factor and neurotensin

10   have blocked N-terminal and no histidine amino acid residues in their sequences); d) structure and function analyses through sequential *in situ* chemical or enzymatic modifications is carried out on the adsorbed analyte with minimal loss at each step of reaction and wash; and e) a probe element with surface bound substrate (e.g., angiotensin I) is used to monitor specific

15   enzyme activity (e.g., trypsin) *in situ* (e.g., inside the gastrointestinal tract of the human body).


2. A solution of horse heart myoglobin (325 pmole, 16,952 Da) is mixed with 50 μl of TSK-SW IDA-Cu(II) at pH 7.0 (20 mM sodium phosphate, 0.5 M sodium chloride) at 23°C for 10 min. The gel is separated from the solution

20   by centrifugation and then washed with 500 μl of buffer two times and 500 μl of water two times. The quantity of remaining myoglobin in all these solutions are then estimated spectrophotometrically, the quantity adsorbed on the gel can then be calculated. The gel is suspended in 50 μl of water and then serially diluted into water. An aliquot of 0.5 μl of the diluted gel

suspension is mixed with 1 µl of sinapinic acid (dissolved in 30% methanol, 0.1% trifluoroacetic acid) and analyzed by laser desorption time-of-flight mass spectrometry. Figure 7 shows that a detectable signal (signal/noise=6, after averaging 50 laser shots) of myoglobin is obtained with a calculated quantity

5    of 4 to 8 fmole deposited on the probe tip.

This example illustrates that affinity adsorbed analytes on a surface are much more easier to transfer and are free from any loss by nonspecific adsorption to container and transfer device surfaces. The adsorbed analyte is sequestered on predetermined areas (that are even less than the laser spot

10   size) of the sample presenting surface in low (atto to femtomole) quantities at a defined surface density or local concentration required for the efficient detection by laser desorption/ionization time-of-flight mass spectrometry.

3.   The human β casein peptides (E2-K18) are synthesized on an Applied Biosystem Model 430A Peptide Synthesizer using the NMP-HOBt protocol.

15   The Ser residues to be phosphorylated are coupled to the peptide chain without side chain protecting group. The unprotected Ser are first phosphinylated using di-t-butyl-N,N,-diisopropyl-phosphoramidite. The phosphite ester is then oxidized with t-butyl peroxide, washed, and cleaved from the resin. All the side chain protecting groups are removed with 95%

20   trifluoroacetic acid. The crude phosphopeptides are extracted with methyl t-butyl ether and dried. This crude preparation of synthetic phosphopeptides is dissolved in 50 mM MES, 0.15 M sodium chloride, pH 6.5 and mixed with 50 µl of tris(carboxymethyl)-ethylenediamine (TED)-Fe(III) immobilized on porous Sepharose (synthesized as described by Yip, T.-T. and Hutchens, T.W.,

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*Protein Expression and Purification* 2: 355-362 (1991), ligand density 65

μmole/ml) at 23°C for 15 min. The gel is washed with 500 μl of the same

buffer three times and then with 500 μl of water once. An aliquot of 1 μl of

gel is mixed with 1 μl of sinapinic acid (dissolved in 30% methanol, 0.1%

5      trifluoroacetic acid) on the probe tip and analyzed by laser desorption time-of-

flight mass spectrometry. After acquiring five spectra (average of 100 laser

shots per spectrum) on various spots of the probe tip, the sinapinic acid is

removed by methanol, and the remaining phosphopeptides adsorbed on TED-

Fe(III) is digested directly on the probe tip by 1 μl of alkaline phosphatase

10     (ammonium sulfate suspension, Sigma) in 50 mM HEPES pH 7.0 at 23°C for

10 min. in a moist chamber. The gel is washed with water to remove enzyme

and salt. Sinapinic acid is added and the sample is reanalyzed by laser

desorption time-of-flight mass spectrometry. Figure 8 (top profile) shows the

distribution of casein peptide (1934 Da) with multiple phosphorylated forms.

15     After *in situ* alkaline phosphatase digestion, only the original

nonphosphorylated form remains (lower profile).

This example illustrates the application of SEAC as a quick monitor

of phosphopeptide synthesis in a crude mixture without prior cleanup. The

identity of the phosphopeptide is readily confirmed by *in situ* alkaline

20     phosphatase digestion.


4. Aliquots of 100 μl of preterm infant formula (SIMILAC, Meade Johnson)

and gastric content of preterm infant aspirated 90 min after feeding of the

formula are mixed with 50 μl of TED-Fe(III) Sepharose in 0.1 M MES, 0.15

M sodium chloride, pH 6.5 at 23°C for 15 min. The gel is washed with 500

μl of the same buffer three times and then with 500 μl of water once.
Aliquots of 1 μl of gel suspensions or preterm infant formula or gastric
aspirate are mixed with 2 μl of sinapinic acid (dissolved in 50% acetonitrile,
0.1% trifluoroacetic acid) on the probe tip and analyzed by laser desorption

5       time-of-flight mass spectrometry. Figure 9 shows that the mass spectrum of
whole gastric aspirate (second from top profile) is quite similar to that of
whole infant formula (bottom profile) in the 1,000-15,000 Da region.
However, the mass spectra of analytes selectively adsorbed by TED-Fe(III)
from the two samples are quite different, there are more low molecular

10      weight phosphopeptides (i.e., bound by TED-Fe(III)) present in the gastric
aspirate (top profile) than in the formula (second from bottom profile) due to
the gastric proteolytic digestion of phosphoproteins present in the formula.

This example illustrates that SEAC is particularly useful in analyzing
specific analytes in biological samples. Phosphopeptides are more difficult to

15      detect in the presence of other contaminating components in a complex
sample because they are less ionized in the positive ion mode. However,
when the phosphopeptides are selectively adsorbed and all other components
in the sample are removed, no such signal depression occurs.

5. Aliquots of 200 μl of human and bovine histidine-rich glycoprotein are

20      mixed with 50 μl of IDA-Cu(II) Sepharose (Pharmacia) at pH 7.0 (20 mM
sodium phosphate, 0.5 M sodium chloride) at 23°C for 10 min. The gel is
washed with 500 μl buffer two times and 500 μl water once. Aliquots of 1 μl
of gel are mixed with 2 μl of sinapinic acid (dissolved in 30% methanol 0.1%
trifluoroacetic acid) and analyzed by laser desorption time-of-flight mass

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spectrometry. After acquiring five spectra (average of 100 laser shots per spectrum) on various spots of the probe tip, the sinapinic acid is removed by methanol wash. The remaining glycoproteins adsorbed on the IDA-Cu(II) gel is then digested with N-glycanase in 20 mM sodium phosphate, 0.5 M sodium

5    chloride, 3 M urea, pH 7.0 at 37°C overnight in a moist chamber. After washing with water to remove enzyme and salt, 2 μl of sinapinic acid is added and the sample is analyzed by mass spectrometry. After acquiring five spectra (average of 100 laser shots per spectrum) on various spots of the probe tip, the sinapinic acid is removed by methanol. Aliquots of 2 μl of

10   trypsin in 0.1 M sodium bicarbonate are added and incubated at 37 °C for 30 min in a moist chamber. After a water wash to remove enzyme and salt, sinapinic acid is added and the sample is analyzed by mass spectrometry. After acquiring five spectra (average of 100 laser shots per spectrum) on various spots of the probe tip, the sinapinic acid is removed by methanol.

15   Aliquots of 2 μl of 20 mM CuSO$_4$ is added. This is followed by addition of 2 μl of sinapinic acid and then analyses by mass spectrometry. After acquiring five spectra (average of 100 laser shots per spectrum) on various spots of the probe tip, the sinapinic acid is removed by methanol. Aliquots of 2 μl of diethylpyrocarbonate (Sigma) in 5 mM HEPES, pH 6.5 are added

20   and incubated at 23°C for 30 min. After a water wash to remove chemicals and buffer salts, 2 μl of sinapinic acid is added and the sample is analyzed by mass spectrometry. To obtain a partial sequence of the metal-binding peptides, instead of modifying the histidine residues with diethylpyrocarbonate, add 1 ul of carboxypeptidase Y (Boehringer Mannheim)

25   to the tryptic digest adsorbed on the surface and incubate at room

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temperature in a moist chamber for 5 min. Wash away the enzyme and salt

with water, add 1 ul of sinapinic acid and analyze by mass spectrometry.

Figure 10A shows the composite mass spectra of human and bovine histidine-

rich glycoprotein adsorbed on IDA-Cu(II) Sepharose before and after N-

5      glycanase digestion. The mass shifts represent the removal of carbohydrate

from the respective glycoproteins. Figure 10B shows the composite mass

spectra of trypsin digested peptides from the deglycosylated proteins of the

two species (top profile for human protein, second from bottom profile for

bovine protein) and *in situ* Cu(II)-binding of the trypsin digested peptides of

10     the two species (second from top profile for human protein, bottom profile for

bovine protein; the numbers 1, 2 indicate the number of copper bound).

Figure 10C shows that one such Cu(II)-binding peptide (bottom profile) has

at least 4 His residues which are specifically modified by diethylpyrocarbonate

to form 4 N-carbethoxy-histidyl adducts (1-4, top profile). Figure 10D shows

15     the partial C-terminal sequence of the major Cu-binding peptide in the bovine

histidine rich glycoprotein. This example illustrates the effective use of SEAC

to probe the structure and function of metal-binding domains of proteins from

different species.


II. Surface immobilized antibody as the affinity capture device

20     1. Polyclonal rabbit anti-human lactoferrin antibody is custom generated

against purified human lactoferrin by Bethyl Laboratories (Montgomery, TX).

The antibody is affinity-purified by thiophilic adsorption and immobilized

lactoferrin columns. Sheep anti-rabbit IgG covalently attached to magnetic

beads are obtained from Dynal AS, Oslo, Norway (uniform 2.8 μm

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supermagnetic polystyrene beads, ligand density 10 µg sheep IgG per mg bead). Human lactoferrin (1 nmole, [69]Fe-labeled, 81,100 Da) is incubated with rabbit anti-human lactoferrin antibody in 20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.0 at 37 °C for 30 min. Subsequently, 40 µl of sheep

5  anti-rabbit IgG on Dynabeads (6-7 x 10^{8} beads/ml) is added and incubated at 37°C for 30 min. The beads are washed with 500 µl of sodium phosphate buffer three times and 500 µl water two times. The final amount of human lactoferrin bound to the complex is estimated to be 4 pmole. Approximately one-tenth of the beads is transferred to a teflon-coated magnetic probe tip,

10  mixed with 2 µl of sinapinic acid (dissolved in 30% methanol, 0.1% trifluoroacetic acid) and analyzed by laser desorption time-of-flight mass spectrometry. Figure 11 shows the presence of lactoferrin (81,143 Da) in the antigen-primary antibody-secondary antibody complex (upper profile), whereas the primary antibody-secondary antibody control (lower profile)

15  shows only the rabbit antibody signal (149,000 Da for singly charged, and 74,500 Da for the doubly charged).

This example illustrates that a) laser desorption is successfully carried out on analyte affinity-adsorbed on surface immobilized antibody (if the analyte signal is unambiguously identified in a mixture of primary antibody-analyte

20  complex, any capture device, e.g., surface immobilized secondary antibody, Protein A, Protein G, Streptavidin, of the primary antibodies is used in this method of identifying the analyte); b) the principle of protein discovery via specific molecular recognition events where one of the analytes is detected through its association with the primary target of capture; and c) the use of

25  magnetic surface as efficient capture device.

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2. Affinity-purified rabbit anti-human lactoferrin is covalently bound to the tip of an activated nylon probe element (2 mm diameter) via glutaraldehyde. This is immersed in 1 ml of preterm infant urine, pH 7.0, containing 350 fmole of human lactoferrin and stirred at 4-8 °C for 15 hr. The nylon probe

5    tip is removed and washed with 1 ml of  20 mM sodium phosphate, 0.5 M sodium chloride, 3 M urea, pH 7.0 three times and 1 ml of water two times. An aliquot of 2 µl of sinapinic acid (dissolved in 30% methanol, 0.1% trifluoroacetic acid) is added and the sample is analyzed by laser desorption time-of-flight mass spectrometry.  Figure 12 shows the human lactoferrin

10   molecular ion (signal/noise=2.5, average of 25 laser shots) in the mass spectrum.  Figure 13 shows the equivalent mass spectrum of whole preterm infant urine containing 1 nmole/ml of lactoferrin; the signal suppression caused by the presence of other components in the urine sample is so severe that even addition of several thousand fold excess over 350 fmole/ml of

15   lactoferrin as described for Figure 12 can not be detected.

      This example illustrates the use of a SEAC device on a flat surface (a two-dimensional configuration) of a flexible probe element. This SEAC device may be used to isolate target analyte materials from undifferentiated biological samples such as blood, tears, urine, saliva, gastrointestinal fluids,

20   spinal fluid, amniotic fluid, bone marrow, bacteria, viruses, cells in culture, biopsy tissue, plant tissue or fluids, insect tissue or fluids, etc. The specific affinity adsorption step cleaned up the analyte from contamination by other components in a complex sample and thus overcome the signal depression effect especially when the analyte is present in very low concentration

25   (femtomole/ml).

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3. Further improvement of detection sensitivity by the SEAC technique is

achieved by amplification of a label bound to the analyte. One way of doing

this is by the combination of enzyme catalysis and the streptavidin-biotin

system. After capturing minute quantities of lactoferrin on a nylon probe

5      element as described in Example 3.II.2. biotinylated anti-lactoferrin antibody

or biotinylated single-stranded DNA is used to bind specifically to the

lactoferrin. Streptavidin is then added to bind specifically to the biotinylated

label. Finally biotinylated alkaline phosphatase is added to bind specifically

to the streptavidin. Since several such biotinylated alkaline phosphatase can

10     bind to one streptavidin, there is a primary level of amplification. The second

level of amplification comes from the enzyme catalysis where the enzyme can

achieve a turnover number of $10^2$ to $10^3$ min$^{-1}$. Assay of alkaline phosphatase

enzyme activity can easily be accomplished by using a low molecular weight

phosphorylated substrate such as ATP, NADPH or a phosphopeptide. The

15     efficiency of detecting the mass shift of a low molecular weight analyte is

much higher than that of a 80 kDa glycoprotein.


4. The ultimate improvement of detection at the present moment is achieved

by the amplification based on the polymerase chain reaction principle. After

capturing minute quantities of lactoferrin on a nylon probe element as

20     described in Example 3.II.2. biotinylated anti-lactoferrin antibody or

biotinylated single-stranded DNA is used to bind specifically to the

lactoferrin. Streptavidin is then added to bind specifically to the biotinylated

label. A piece of biotinylated linear DNA is finally added to bind to the

streptavidin. This bound DNA label is amplified in a 30-cycle polymerase

chain reaction procedure. Each cycle consists of a 1 min denaturation step at 94°C, a 1 min annealing reaction at 58°C, and a 1 min primer extension reaction at 72°C. This technique provides amplification factors in the $10^6$ fold range. The amplified DNA is detected directly by laser desorption mass

5      spectrometry using 3-OH picolinic acid as the matrix.

5. Polyclonal rabbit anti-bovine histidine rich glycoprotein antibody is custom generated against purified bovine histidine rich glycoprotein by Bethyl Laboratories (Montgomery, TX). The antibody is affinity-purified by

10     thiophilic adsorption and immobilized bovine histidine rich glycoprotein columns. The purified antibody is immobilized on AffiGel 10 (BioRad Laboratories, Hercules, CA, ligand density 15 µmole/ml gel) according to manufacturer's instruction. An aliquot of 200 µl of bovine colostrum is diluted with 200 µl of 20 mM sodium phosphate, pH 7.0 and mixed with 50

15     µl of immobilized antibody at 23°C for 30 min. The gel is washed with 500 µl of 20 mM sodium phosphate, 0.5 M sodium chloride, 3 M urea, pH 7.0 three times and 500 µl of water two times. An aliquot of 1µl of the washed gel is mixed with 2 µl of sinapinic acid (dissolved in 30% methanol, 0.1% trifluoroacetic acid) on the probe tip and analyzed by laser desorption time-of-

20     flight mass spectrometry. Figure 14 shows the composite mass spectra of purified bovine histidine rich glycoprotein (lower profile) and proteins affinity adsorbed from bovine colostrum (upper profile). The result indicates the presence of intact histidine rich glycoprotein and its major proteolytic fragments in bovine colostrum.

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This example illustrates the effective use of SEAC as a fast and simple technique to detect and characterize new proteins in a small quantity of biological fluid. This result supports the initial findings obtained by the very labor-intensive technique of immunoblotting of polyacrylamide gel

5    electrophoresis.

6. Antibody epitope mapping is easily achieved with the SEAC technique. Three different sources of anti-human follicle stimulating hormone (a polyclonal specific against beta FSH from Chemicon International, Temecula, CA, a monoclonal specific against beta 3 epitope from Serotec, Indianapolis,

10   IN, a monoclonal from Biodesign, Kennebunk, ME) are immobilized on AffiGel 10 according to manufacturer's instruction. These immobilized antibodies are all tested to bind specifically the follicle stimulating hormone by incubating with two different preparations of follicle stimulating hormone (a semipure preparation from Chemicon, and a crude preparation from

15   Accurate Chemical and Scientific Corp.) and then analyzed by mass spectrometry in the presence of sinapinic acid. Then the semipure preparation of human FSH (Chemicon) is digested with trypsin and separate aliquots (7 ul) are reacted with the immobilized antibodies (10 ul of 1:1 gel suspension) in phosphate-buffered saline at 4°C for 2 hr. After washing with

20   phosphate-buffered saline and water, the adsorbed proteins are analyzed by laser desorption mass spectrometry in the presence of sinapinic acid. Figure 15 shows the composite mass spectra of the peptides of follicle stimulating hormone recognized by the different antibodies. The two monoclonal antibodies clearly recognize different epitopes, whereas the polyclonal

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recognizes multiple epitopes common to those recognized by both monoclonals.


III. Surface immobilized nucleic acid as the affinity capture device

1. Single-stranded DNA immobilized on 4% agarose beads are obtained from

5      GIBCO BRL (Gaithersburg, MD, ligand density 05-1.0 mg DNA/ml gel). An

aliquot of ^{125}I-human lactoferrin (equivalent to 49 nmole) is mixed with 100

µl of immobilized single-stranded DNA in 20 mM HEPES, pH 7.0 at room

temperature for 10 min. The gel is washed with 500 µl of HEPES buffer five

times and then suspended in equal volume of water. The amount of

10     lactoferrin bound per bead is estimated to be 62 fmole by determining the

radioactivity and counting the number of beads per unit volume. Various

numbers of beads (from 1 to 12) are deposited on 0.5 mm diameter probe tips,

mixed with 0.2 µl of sinapinic acid (dissolved in 30% methanol, 0.1%

trifluoroacetic acid) and analyzed by laser desorption time-of-flight mass

15     spectrometry. Figure 16 shows the mass spectrum of lactoferrin affinity

adsorbed on a single bead of single-stranded DNA agarose. This is a

representative spectrum from a total of five (average of 100 laser shots per

spectrum) obtained from the single bead.

This example illustrates that laser desorption is successfully carried out

20     on analyte affinity adsorbed on surface immobilized biopolymer such as

nucleic acid. The specificity of interaction between human lactoferrin and

DNA has been documented and effectively exploited in capturing minute

quantities of lactoferrin from preterm infant urine. In this case, the

combination of the efficiency of transferring the lactoferrin affinity capture

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device with the sensitivity of laser desorption mass spectrometry greatly increases the sensitivity of detection.

2. An aliquot of 1 ml of preterm infant urine containing 30 pmole of $^{59}$Fe-human lactoferrin is mixed with 20 μl of single-stranded DNA agarose in 0.1

5    M HEPES pH 7.4 at 23 °C for 15 min. The gel is washed with 500 μl of HEPES buffer two times and 500 μl of water two times. The gel is suspended in equal volume of water and 1 μl of the suspension (containing not more than 350 fmole of adsorbed lactoferrin as determined by radioactivity) is mixed with 1 μl of sinapinic acid (dissolved in 30% methanol, 0.1%

10   trifluoroacetic acid) on a probe tip and analyzed by laser desorption time-of-flight mass spectrometry. Figure 17 shows the mass spectrum of lactoferrin extracted from urine by surface immobilized DNA as the affinity capture device.

This example illustrates the efficiency and sensitivity of detecting

15   minute quantities of high molecular weight analyte in biological fluid with the DNA capture device.

IV. Surface immobilized miscellaneous biomolecule as the affinity capture device

1. Soybean trypsin inhibitor (Sigma) is immobilized on AffiGel 10 (BioRad)

20   according to manufacturer's instructions. An aliquot of 100 μl of human duodenal aspirate is mixed with 50 μl of surface immobilized soybean trypsin inhibitor at pH 7.0 (20 mM sodium phosphate, 0.5 M sodium chloride) at 23°C for 15 min. The gel is then washed with 500 μl of phosphate buffer three

times and 500 µl of water two times. Aliquots of 1 µl of gel suspension or the

original duodenal aspirate are mixed with 2 µl of sinapinic acid (dissolved in

50% acetonitrile, 0.1% trifluoroacetic acid) and analyzed by laser desorption

time-of-flight mass spectrometry. Figure 18A shows the composite mass

5       spectra of the total duodenal aspirate (lower profile) and the proteins

adsorbed by surface immobilized soybean trypsin inhibitor (upper profile).

The major peak in the affinity captured sample represents trypsin. Similar

results are obtained with only 1 µl of duodenal fluid deposited on a) the tip

of a nylon probe element coupled to soybean trypsin inhibitor via

10      glutaraldehyde and b) the tip of an acrylic probe element coated with

polyacrylamide coupled to soybean trypsin inhibitor via either glutaraldehyde

or divinyl sulfone (Figure 18B).

     These results indicate a) the unambiguity in detecting and

characterizing a specific analyte in biological fluids and b) the feasibility of

15      *in situ* sampling by inserting a flexible (e.g. nylon) probe element through an

endoscope directly into the human body (e.g. small intestine) for diagnostic

purposes.


2.      Streptavidin immobilized on Dynabead (uniform, 2.8 µm,

superparamagnetic, polystyrene beads) is obtained Dynal, AS, Oslo, Norway.

20      Aliquots of 150 µl of human plasma or urine containing 18 pmole of

biotinylated insulin (Sigma) are mixed with 20 µl suspension of streptavidin

Dynabead at pH 7.0 (20 mM sodium phosphate, 0.5 M sodium chloride) at

23°C for 10 min. The beads are then washed with 500 µl buffer containing

3M urea three times and 500 µl water once. Aliquots of 0.5 µl of the bead

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suspension are mixed with 2 μl of sinapinic acid (dissolved in 30% methanol, 0.1% trifluoroacetic acid) and analyzed by laser desorption time-of-flight mass spectrometry. Figure 19A shows the mass spectrum of biotinylated insulin affinity adsorbed from urine. The multiple peaks represent insulin

5    derivatized with one to three biotin groups. Figure 19B shows the mass spectrum of biotinylated insulin affinity adsorbed from plasma.

This example illustrates that laser desorption is carried out on analyte affinity adsorbed via the biotin-streptavidin binding. In view of the tight binding between biotin and avidin (Ka=$10^{15}$ M$^{-1}$), this system serves as an

10   ideal SEAC device for proteins and nucleic acid on a probe surface where *in situ* sequential chemical and enzymatic modifications are performed.

3. Human estrogen receptor DNA-binding domain (84 residues) is expressed in bacteria. The plasmid expression vector pT₇ERDBD (J. Schwabe, MRC Laboratory of Molecular Biology, Cambridge, UK) is transformed into E. coli

15   BL21(DE3)plyS cells (Novagene). Expression of the DNA binding domain is induced by 1 mM isopropylthiogalactoside (GIBCO BRL) and the bacteria are harvested after induction for 3 hr. Whole induced bacteria are analyzed directly by matrix-assisted laser desorption/ionization mass spectrometry to verify that the DNA-binding domain is the major peptide synthesized. The

20   peptide is purified by reverse phase HPLC from the bacterial lyzate, and immobilized on AffiGel 10 (BioRad). A 30-bp DNA sequence containing the estrogen response element is synthesized by Genosys (Houston, TX). Interaction of surface affinity adsorbed apo-, Zn- and Cu-bound forms of DNA-binding domain with sequence specific nucleic acid (estrogen response

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element) are studied on glass probe elements using 3-hydroxypicolinic acid as the matrix.

This example illustrates the use of protein surface functional domain as capture device in SEAC. The effect of metal-binding on the structure and function of such protein surface domains can be investigated.

4. Different aliquots of lectins immobilized on surfaces (e.g., Con A-Sepharose, wheat germ lectin-Sepharose, Pharmacia) are used to bind the glycopeptides in human and bovine histidine-rich glycoprotein tryptic digests. After washing with buffers and water to remove unbound peptides, sequential enzyme digestion are performed *in situ* with FUCase I, MANase I, HEXase I , NANase III and PNGase (Glyko, Inc, Novato, CA). The samples are analyzed with laser desorption time-of-flight mass spectrometry to study the carbohydrate composition of the glycopeptides in the two proteins. This example illustrates the use of SEAC device to tether a glycopeptide, the carbohydrate component of which can then be sequenced *in situ*.

V. Surface immobilized dye as the affinity capture device

Cibacron Blue 3GA-agarose (Type 3000, 4% beaded agarose, ligand density 2-5 μmoles/ml gel) is obtained from Sigma. An aliquot of 200 μl of human plasma is mixed with 50 μl of surface immobilized Cibacron Blue at pH 7.0 (20 mM sodium phosphate, 0.5 M sodium chloride) at 23°C for 10 min. The gel is then washed with 500 μl of buffer three times and 500 μl of water two times. An aliquot of 1 μl of gel suspension is mixed with 2 μl of sinapinic acid (dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid) and analyzed by

laser desorption time-of-flight mass spectrometry. Figure 20 shows the selective adsorption of human serum albumin (doubly charged ion $[M+2H]^{2+}$, 32,000 m/z, singly charged ion $[M+H]^+$, 64,000 m/z, dimer ion, $2[M+H]^+$, 128,000 m/z) from the serum sample by surface immobilized Cibacron Blue

5    (lower profile). Other immobilized dyes tested included Reactive Red 120-agarose, Reactive Blue-agarose, Reactive Green-agarose, Reactive Yellow-agarose (all from Sigma) and each selects different proteins from human plasma.

## EXAMPLE 4

10    ### Surface Enhanced Neat Desorption (SEND)

This example describes the method for desorption and ionization of analytes in which the analyte is not dispersed in a matrix crystalline structure but is presented within, on or above an attached surface of energy absorbing molecules in a position where it is accessible and amenable to a

15    wide variety of chemical, physical and biological modification or recognition reactions. The surface is derivatized with the appropriate density of energy absorbing molecules bonded (covalently or noncovalently) in a variety of geometries such that mono layers and multiple layers of attached energy absorbing molecules is used to facilitate the desorption of analyte molecules

20    of varying masses.

The Examples shown below (Groups I-IV) demonstrate the combined SEND and SEAC where the adsorbed (bonded) energy absorbing molecules also act as affinity adsorption reagents to enhance the capture of analyte molecules.

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I.  Energy absorbing molecules bound by covalent bond to the surface

1.  Cinnamamide (Aldrich) (not a matrix at laser wavelength of 355 nm by prior art) is dissolved in isopropanol: 0.5 M sodium carbonate (3:1) and mixed with divinyl sulfone (Fluka, Ronkonkoma, NY) activated Sepharose

5      (Pharmacia) at 23°C for 2 hr.  The excess energy absorbing molecules are washed away with isopropanol.  The proposed molecular structure is presented in Figure 21.  Aliquots of 2 μl of the bound or free molecules are deposited on the probe tips, 1 μl of human estrogen receptor dimerization domain in 0.1% trifluoroacetic acid is added on top and analyzed by laser

10     desorption time-of-flight mass spectrometry.  The result shows that peptide ion signals are detected only on the bound energy absorbing molecule surface (Figure 20, top profile), the free molecules are not effective (Figure 20, bottom profile).

2.  Cinnamyl bromide (Aldrich) (not a matrix at laser wavelength of 355 nm

15     by prior art) is dissolved in isopropanol:0.5 M sodium carbonate (3:1) and mixed with divinyl sulfone (Fluka) activated Sepharose at 23°C for 15 hr. The excess energy absorbing molecules are washed away with isopropanol. The proposed molecular structure is presented in Figure 23.  Aliquots of 2 μl of the bound or free molecules are deposited on the probe tips, 1 μl of peptide

20     mixtures in 0.1% trifluoroacetic acid is added on top and analyzed by laser desorption time-of-flight mass spectrometry.  The result shows that peptide ion signals are detected only on the bound energy absorbing molecule surface (Figure 24, top profile), the free molecules are not effective (Figure 24, bottom profile).

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3. Dihydroxybenzoic acid is activated by dicyclohexylcarbodiimide and mixed with Fmoc-MAP 8 branch resin (Applied Biosystems, Forster City, CA) at 23°C for 15 hr. The excess energy absorbing molecules are washed away by methanol. The proposed molecular structure is presented in Figure 25.

5      Aliquots of 1 μl of the MAP 8 branch surface with and without bound energy absorbing molecules are deposited on the probe tips, 1 μl of peptide mixtures in 0.1% trifluoroacetic acid was added on top and analyzed by laser desorption time-of-flight mass spectrometry. The result shows that peptide ion signals are detected only on the surface with bound energy absorbing molecules

10    (Figure 26, bottom profile), the control surface without any energy absorbing molecules is not effective (Figure 24, top profile).

4. α-cyano-4-hydorxycinnamic acid is dissolved in methanol and mixed with AffiGel 10 or AffiGel 15 (BioRad) at various pHs at 23°C for 2-24 hours. The excess energy absorbing molecules are washed away by methanol. Aliquots

15    of 2 μl of the bound molecules are deposited on the probe tips, 1 μl of peptide mixtures or myoglobin, or trypsin or carbonic anhydrase is added on top and analyzed by laser desorption time-of-flight mass spectrometry. The result shows that myoglobin ion signal is detected on the surface with bound energy absorbing molecules (Figure 27A) with very little contaminating low mass ion

20    signals (Figure 27B).

5. A 40% polyacrylamide solution is prepared and cast into the desired shape of a probe tip. The gel is allowed to air dry until no noticeable reduction in size is observed. The tip is submerged into a 9% glutaraldehyde / buffer (v/v)

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solution and incubated with gentle shaking at 37°C for 2 hours. After incubation, buffer is used to rinse off excess glutaraldehyde. The activated tip is added to a saturated buffered energy absorbing molecule solution and incubated at 37°C (approx.) for 24 hours (approx.) with gentle shaking.

5    Organic solvents are used to solubilize the energy absorbing molecules in situations that required it. The tip is rinsed with buffer and placed into a 9% ethanolamine/water (v/v) solution to incubate at 25°C with gentle shaking for 30 minutes. Next, the tip is rinsed with buffer and added to a 5 mg/mL solution of sodium cyanoborohydride / buffer to incubate at 25°C for 30

10    minutes. Finally, the tip is rinsed well with buffer and stored until use. The same reaction is carried out on nylon tips which is prepared by hydrolysis with 6N HCl under sonication for 2 minutes and then rinsed well with water and buffer. The same reaction is also performed on acrylic tips activated by soaking in 20% NaOH for 7 days with sonication each day for 30-60 min and

15    then washed. The proposed general molecular structure of the surface is shown in Figure 28.

6. A 40% polyacrylamide solution is prepared and cast into the desired shape of a probe tip. The gel is air dried until no noticeable reduction in size is observed. A 0.5 M sodium carbonate buffer with a pH of 8.8 is prepared as

20    rinsing buffer. The tip is next placed into a solution of divinyl sulfone (Fluka) and buffer at a ratio of 10:1, respectively and incubated for 24 hours. The tip is rinsed with buffer and placed into an energy absorbing molecule buffered solution at a pH of 8 to incubate for 2 hours. The same reaction is carried out on nylon tips which is prepared by hydrolysis with 6N HCl under

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sonication for 2 minutes and then rinsed well with water and buffer. The same reaction is also performed on acrylic tips activated by soaking in 20% NaOH for 7 days with sonication each day for 30-60 min and then washed. The proposed general molecular structure of the surface is shown in Figure

5     29.


7. A 40% polyacrylamide solution is prepared and cast into the desired shape of a probe tip. The gel is air dried until no noticeable reduction in size is observed. An energy absorbing molecule solution at 100 mg/mL in dichloromethane / NMP (2:1 respectively) and a 1M dicyclohexylcarbodiimide/

10    NMP solution are mixed at a ratio of 1:2 (EAM : DCC), respectively. The EAM / DCC solution is next incubated at 25°C for 1 hour while stirring. After incubation, a white precipitate is observed. The white precipitate is filtered in a sintered glass filter. The flow through is the DCC activated EAM. Next, the tip is placed into the DCC activated EAM solution and incubated at 25°C

15    for 2 hours (approx.). The tip is finally rinsed with a variety of solvents such as acetone, dichloromethane, methanol, NMP, and hexane. The same reaction is carried out on nylon tips which is prepared by hydrolysis with 6N HCl under sonication for 2 minutes and then rinsed well with water and buffer. The same reaction is also performed on acrylic tips activated by soaking in

20    20% NaOH for 7 days with sonication each day for 30-60 min and then washed. The proposed general molecular structure of the surface is shown in Figure 30.

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8. A 40% polyacrylamide solution is prepared and cast into the desired shape of a probe tip. The gel is air dried until no noticeable reduction in size was observed. A 100 mg/mL solution of N-α-Fmoc-N-ε-Fmoc-L-lysine in dichloromethane / NMP (2:1 respectively) and a 1M DCC / NMP solution are

5      mixed at a ratio of 1:2 (lysine : DCC), respectively. The lysine / DCC solution is incubated at 25°C for 1 hour while stirring. After incubation, a white precipitate is observed and filtered with a sintered glass filter. The flow through is DCC activated lysine. The tip is placed into the DCC activated lysine solution and incubated at 25°C for 2 hours (approx.). The tip is next

10     placed into 5mL of piperidine and incubated at 25°C for 45 minutes with gentle stirring. DCC activated lysine is repeatedly reacted in consecutive cycles with the tip until the desired lysine branching is attained. An EAM solution at 100 mg/mL in dichloromethane / NMP (2:1 respectively) and a 1M DCC/NMP solution are mixed at a ratio of 1:2 (EAM : DCC), respectively.

15     The EAM / DCC solution is incubated at 25°C for 1 hour while stirring. After incubation, a white precipitate is observed and filtered with a sintered glass filter. The flow through is the DCC activated EAM. The EAM contains an acid functional group that reacts with the DCC. The tip is placed into the DCC activated EAM solution and incubated at 25°C for 2 hours (approx.) with

20     gentle shaking. Finally, the tip is rinsed with excess dichloromethane, NMP, and methanol before use. The same reaction is carried out on nylon tips which is prepared by hydrolysis with 6N HCl under sonication for 2 minutes and then rinsed well with water and buffer. The same reaction is also performed on acrylic tips activated by soaking in 20% NaOH for 7 days with

sonication each day for 30-60 min and then washed. The proposed general

molecular structure of the surface is shown in Figure 31.

II. Energy absorbing molecules bound by co-ordinate covalent bond to the

surface

5        1. Thiosalicylic acid (Aldrich) is dissolved in either water or 50% methanol

in water or methanol. These solutions are either used as such or the pH of

the solutions is adjusted to 6.5 with 0.5 M sodium bicarbonate or ammonium

hydroxide or triethylamine. Cu(II) ion are chelated by either iminodiacetate

(IDA) (Chelating Sepharose Fast Flow, Pharmacia) or

10       tris(carboxymethyl)ethyleneidamine (TED) (synthesized as described by Yip

and Hutchens, 1991) immobilized on gel surface. The solutions of energy

absorbing molecule are mixed with the IDA-Cu(II) or TED-Cu(II) gel at 4° to

23°C for 5 min to 15 hours. The excess energy absorbing molecules are

washed away with either water or 50% methanol in water or methanol. The

15       proposed molecular structure of the surface is shown in Figure 32. Aliquots

of 1μl of the bound energy absorbing molecules are deposited on the probe

tips, 1 μl of peptide mixtures or estrogen receptor dimerization domain or

myoglobin in 0.1% trifluoroacetic acid is added on top and analyzed by laser

desorption time-of-flight mass spectrometry. Figure 33 shows one

20       representative mass spectrum of estrogen receptor dimerization domain

desorbed from this surface.

2. Sequential *in situ* reactions are readily accomplished on samples deposited

on top of an EAM surface. Thiosalicylic acid co-ordinate covalently bound to

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IDA-Cu(II) on a probe surface is prepared as described in Section 2.1. An aliquot of 1 µl of (GHHPH)₅G peptide is deposited on the surface and analyzed by laser desorption time-of-flight mass spectrometry. After obtaining several spectra (each an average of 50 laser shots), the sample is

5   removed. An aliquot of 2 µl of carboxypeptidase Y (Boehringer Mannheim) is added directly on the surface and incubated at 37°C in a moist chamber for 5 min to 1 hr. The *in situ* enzyme digestion is terminated by 1 µl of 0.1% trifluoroacetic acid and the sample is reanalyzed by mass spectrometry.

3. Another illustration of sequential *in situ* reaction is trypsin digestion

10  followed by C-terminal sequencing. Thiosalicylic acid co-ordinate covalently bound to IDA-Cu(II) on a probe surface is prepared as described in Section 2.1. An aliquot of 1 µl of estrogen receptor dimerization domain (6168.4 Da) is deposited on the surface and analyzed by laser desorption time-of-flight mass spectrometry. After obtaining several spectra (each an average of 20

15  laser shots), the sample is removed. An aliquot of 2 µl of trypsin (Sigma) in 0.1M sodium bicarbonate is added on the surface and incubated at 37°C for 15 min. The *in situ* enzyme digestion is terminated by 1 µl of 0.1% trifluoroacetic acid and the sample is reanalyzed by mass spectrometry. After obtaining several spectra (each an average of 20 laser shots), the sample is

20  removed. An aliquot of 2 µl of carboxypeptidase Y (Boehringer Mannheim) is added directly on the surface and incubated at 37°C in a moist chamber for 1 hr. The *in situ* enzyme digestion is terminated by 1 µl of 0.1% trifluoroacetic acid and the sample is reanalyzed by mass spectrometry.

III.    Energy absorbing molecules bound by ionic bond to the surface

        Sinnapinic acid or α-cyano-4-hydroxycinnamic acid are suspended in

water and the pH is adjusted to 6.6 with dilute sodium hydroxide. Tentacle

DEAE Fractogel (EM Separations, Gibbstown, NJ) is washed with 20 mM

5       HEPES, pH 6.0 and suction dried. The energy absorbing molecules solution

is mixed with the DEAE gel at 23°C for 15 hours. The gel is washed with

water until all excess energy absorbing molecules were removed. The

proposed molecular structure of the surface is shown in Figure 34. An aliquot

of 0.5 µl of the bound energy absorbing molecules is deposited on the probe

10      tips, 1 µl of estrogen receptor dimerization domain or myoglobin in 0.1%

trifluoroacetic acid is added on top and analyzed by laser desorption time-of-

flight mass spectrometry. Figures 35 A and B show the mass spectra.


IV. Energy absorbing molecules bound by hydrophobic/Van der Waals bonds
to the surfaces

15      1.      α-cyano-4-hydroxcinnamic acid is dissolved in 50% methanol in water

and dimethylsulfoxide. This is mixed with aminomethylated polystyrene at

23°C for 15 hours. The excess energy absorbing molecules are washed away

with 50% methanol in water. The proposed molecular structure is shown in

Figure 36. An aliquot of 1 µl of the bound energy absorbing molecules is

20      deposited on the probe tip, 1 µl of peptide is added on top and analyzed by

laser desorption time-of-flight mass spectrometry.


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## EXAMPLE 5

### Surfaces Enhanced for Photolabile Attachment and Release (SEPAR)

The linear assembly of individual building blocks (monomers) that define the structure and characteristics of biopolymers such as DNA, RNA,

5    and protein are often unknown but are decoded or sequenced (in whole or in part) with a method that involves differential mass determinations of partially digested (i.e., chemical or enzymatic) biopolymer analytes by laser desorption/ionization time-of-flight (TOF) mass spectrometry (MS).

Given biopolymers are first coupled to the SELDI probe element

10   surface through one or more (multiple) covalent photolytic (i.e., light sensitive) bonds. Next, various number of individual units (monomers) at the ends of the biopolymer are cleaved (i.e., removed) in a single reaction by enzymatic or chemical methods. The analytes remaining on the probe element surface consist of a variety (population) of mass-defined biopolymers

15   with different numbers of their end monomer units missing. A small but sufficient portion of the modified biopolymers are uncoupled (untethered) from the probe element surface by laser light, that is, by cleavage of the photolytic bonds with UV light between 260 nm and 365 nm. The uncoupled biopolymers are desorbed/ionized by time-of-flight mass spectrometry.

20   I. Coupling of biopolymers to the SELDI surface

Three components are involved: 1) a surface that is activated to react with either amine or carboxyl groups, or both; 2) photolytic compound, typically azo-based compound of the general formula $R_1$-N=N-$R_2$, e.g., 5-(4-aminophenylazo)salicylic acid (Aldrich), azodicarbonamide (Aldrich), or other

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mechanisms generating such photolytic bond such as the active hydrogen
reactive chemistries with diazonium compounds are used; and 3) biopolymer,
e.g., proteins, nucleic acids, carbohydrates.

A photolytic compound must first be attached to activated surface, e.g.,

5        azodicarbonamide to amine-reactive surfaces, aminophenylazosalicylic acid to
either amine or carboxyl reactive surfaces. Then activate either photolytic
compound or biopolymer by one of many conventional chemistries, e.g., amine
reactive chemistries - cyanogen bromide, N-hydroxysuccinimide esters, FMP
activation, EDC-mediated, divinyl sulfone; hydroxyl reactive chemistries -

10       epoxy activation, divinyl sulfone; sulfhydryl reactive chemistries - iodoacetyl
activation, maleimide, divinyl sulfone, epoxy activation; carbonyl reactive
chemistries - hydrazide, reductive amination; active hydrogen reactive
chemistries - diazonium, which also generate a photolytic azo bond at the
same time. Finally, attach the biopolymer to photolytic compound through

15       one or more (multiple) bonds. Wash away the excess chemicals with aqueous
and organic solvents, high ionic strength and low pH solvents in multiple
cycles.


II. Mass spectrometric analysis to verify structural integrity

UV laser from 260 to 365 nm will cleave the photolytic bond. The

20       uncoupled biopolymers are desorbed/ionized by MALDI TOF (one skilled in
the art knows that SEND, SEAC and SEPAR may also be used).

III. *In situ* sequencing of biopolymer

This is accomplished by any of the well-known sequential degradation with enzymatic or chemical methods, e.g., N-terminal sequencing of proteins with aminopeptidase, C-terminal sequencing of proteins with carboxypeptidase, N-terminal sequencing of proteins with Edman degradation; sequencing of nucleic acids with exonuclease, sequencing of nucleic acids with Sanger's method; sequencing of carbohydrate with specific enzymes such as neuraminidase, mannase, fucase, galactosidase, glucosidase, O- or N-glycanase. After washing to remove excess reagent and reaction products, the analytes remaining tethered on the surface via multiple photolytic bonds consisting of a population of mass-defined biopolymers with different numbers of their end monomer missing are analyzed by MALDI TOF MS (one skilled in the art knows that SEND, SEAC and SEPAR may also be used).

Multiple internal sequencing with enzymatic or chemical methods, e.g., cleavage of proteins with endoprotease or cyanogen bromide followed by sequential degradation of N- and/or C-terminal; cleavage of nucleic acids with endonuclease followed by sequential degradation with exonuclease or chemical method; cleavage of polysaccharide chains with endoglycosidase H or endoglycosidase F followed by sequential cleavage with specific enzymes. After washing to remove excess reagent and reaction products, the analytes remaining on the surface consisting of multiple populations of mass-defined biopolymers with different numbers of their end monomer missing are analyzed by MALDI TOF MS (one skilled in the art knows).

IV. Specific Examples of Sequencing

A demonstration of this principle is provided by the actual amino acid

sequence determination of a 26-residue peptide:


GHHPHGHHPHGHHPHGHHPHGHHPHGHHPHG.


5        This peptide (GHHPH)$_5$G defines the metal-binding domain within the

intact sequence of the 80-kDa protein known as histidine-rich glycoprotein

(HRG).

Glass beads with surface arylamine groups as coupling ligands (Sigma) are

washed with and suspended in cold 0.3M HCl. A 50 mg/mL aqueous

10       solution of NaNO$_2$ is added to the beads at a ratio of 1:5 (v/v)

(NaNO$_2$:HCl) and incubated at 4°C for 15 minutes with gentle shaking.

After incubation, the beads are washed with cold 0.3M HCl and 50 mM

sodium phosphate buffer pH 8.0. The peptide to be sequenced is added to

the beads in sodium phosphate buffer at pH 8.0 and incubated for 24 hrs.

15       at 4°C with gentle shaking. The beads with coupled peptides are washed

with sodium phosphate buffer, sodium phosphate buffer with high

concentration of salt (e.g., 1.0 M), dilute acid and organic solvent (e.g.,

methanol) until no peptide signal is detected in the supernate by MALDI-

TOF mass spectrometry (one skilled in the art knows SEND, SEAC, and

20       SEPAR may also be used) or by absorbance at 220 nm. An aliquot of 1 μL

of the beads is then deposited on the probe tip, 1 μL of sinapinic acid

(dissolved in 50% methanol/0.1% trifluoroacetic acid) is mixed with the

beads and the sample was analyzed by laser desorption time-of-flight mass

spectrometry. After obtaining several spectra (each an average of 50 laser

shots), the remaining peptides on the surface are washed free of sinapinic

acid with methanol and then digested with carboxypeptidase Y (Boehringer

Mannheim) at 23°C in a moist chamber. The digested peptides are next

5     washed with phosphate buffered saline (PBS) pH 8.0. An aliquot of 1 μL

of sinapinic acid is added to the surface and analyzed again by laser

desorption time-of-flight mass spectrometry. The result of the C-terminal

sequence analysis of the GHHPHG sequence is shown in Figure 35. A

nascent sequence of the peptide is observed. The sequence is deduced by

10   the differences in the mass between two peaks.

The second example is the simultaneous sequencing of multiple

peptides covalently bound by photolytic bonds to a surface. Human

estrogen receptor dimerization domain (6168.4 Da) is tethered to the

surface via multiple photolytic bonds. The peptide has three methionine

15   residues in its sequence and are cleaved specifically by cyanogen bromide

to generate peptides of masses 2170.58 Da (D1-M18), 3118.77 Da (A19-

M45), 535.62 Da (S46-M50) and 397.62 Da (E51-L53). All these peptides

are bound to the surface via the photolytic bonds. Each of these are

subsequently digested *in situ* with carboxypeptidase Y to generate a

20   nascent sequence that is completely resolved from the other.

Another approach to protein structure determination is

simultaneous N-terminal sequencing of multiple peptides generated by

tryptic digest of a protein coupled to a surface by multiple photolytic

bonds. Insulin B chain is tethered to the surface via multiple photolytic

25   bonds. The peptide has two lysine/arginine residues in its sequence that

are cleaved specifically by trypsin to generate peptides of masses 2585.9 Da
(F1-R22) and 859.0 Da (G23-K29), both of which are bound to the surface
via the photolytic bonds. Each of these are subsequently subjected *in situ*
to either aminopeptidase digestion or multiple cycles of Edman

5      degradation to generate a nascent sequence that is completely resolved
from the other.

         Coupling and sequencing of nucleic acids is performed with similar
procedure. Glass beads with surface arylamine groups as coupling ligands
(Sigma) are washed with and suspended in cold 0.3M HCl. A 50 mg/mL

10     aqueous solution of $NaNO_2$ is added to the beads at a ratio of 1:5 (v/v)
($NaNO_2$:HCl) and incubated at 4°C for 15 minutes with gentle shaking.
After incubation, the beads are washed with cold 0.3M HCl and 50 mM
sodium phosphate buffer pH 8.0. The DNA (e.g., estrogen receptor
responsive element, a 30-base pair oligonucleotide) to be sequenced is

15     added to the beads in sodium phosphate buffer at pH 8.0 and incubated for
24 hrs. at 4°C with gentle shaking. The beads with coupled DNA are
washed with sodium phosphate buffer, sodium phosphate buffer with high
concentration of salt (e.g., 1.0 M), dilute acid and organic solvent (e.g.,
methanol) until no DNA signal is detected in the supernate by MALDI-

20     TOF mass spectrometry (one skilled in the art knows that SEND, SEAC
and SEPAR may also be used) or by absorbance at 260 nm. An aliquot of
1 μL of the beads is then deposited on the probe tip, 1 μL of 3-
hydroxypicolinic acid (dissolved in 50% methanol/0.1% trifluoroacetic acid)
is mixed with the beads and the sample is analyzed by laser desorption

25     time-of-flight mass spectrometry. After obtaining several mass spectra

(each an average of 50 laser shots), the remaining DNA bound on the

surface are washed free of 3-hydroxypicolinic acid with methanol and

digested with exonuclease (Boehringer Mannheim) at 23°C in a moist

chamber. The digested DNA on the surface are next washed with

5      phosphate buffered saline (PBS) pH 8.0 to remove excess reagent and

reaction products. An aliquot of 1 μL of 3-hydroxypicolinic acid is added

to the surface and analyzed again by laser desorption time-of-flight mass

spectrometry. A nascent sequence of the DNA is generated. The sequence

is deduced by the differences in the mass between two peaks.

10     Carbohydrate chains are oxidized by periodate and activated to be

specifically coupled to a photolytic compound on a surface. Sequencing of

the tethered carbohydrate with specific enzymes such as neuraminidase,

mannase, fucase, galactosidase, glucosidase, O- or N-glycanase is carried

out and determined by laser desorption time-of-flight mass spectrometry.

15     5-(4-aminophenylazo)salicylic acid (Aldrich) is coupled to a carboxyl

reactive surface such as arylamine on controlled pore glass beads. The

carbohydrate moieties of human and bovine histidine rich glycoprotein are

oxidized by low concentration (0.2 M) of sodium meta-periodate in water at

23°C for 90 min. The excess reagents are washed away with water. Add

20     the proteins to the 5-(4-aminophenylazo)salicylic acid coupled to controlled

pore glass beads in phosphate buffer, pH 8.0. Then add sodium

cyanoborohydride (0.6 mg/100 μl) and stir in a fume hood at 23°C for 18

hr. Wash extensively with water, 1 M NaCl, and then water again to

remove excess reagents and unreacted proteins. An aliquot of 1 μL of the

25     beads is then deposited on the probe tip, 1 μL of sinapinic acid (dissolved

in 50% methanol/0.1% trifluoroacetic acid) is mixed with the beads and the

sample is analyzed by laser desorption time-of-flight mass spectrometry.

The remaining proteins bound on the surface are washed free of sinapinic

acid with methanol and incubated with 2 μl of trypsin in phosphate buffer

5    pH 8.0 at 37°C for 30 min.  The surface with bound glycopeptides is

washed thoroughly with phosphate buffered saline and water to remove

excess reagent and unbound peptides.  An aliquot of 1 μL of sinapinic acid

is mixed with the beads and the sample is analyzed by laser desorption

time-of-flight mass spectrometry.  After obtaining several mass spectra

10   (each an average of 50 laser shots), the remaining glycopeptides on the

probe surface are washed free of sinapinic acid with methanol and digested

in sequence or in combination with N-acetylneuraminidase (NANase III,

Glyko, 50 mM sodium phosphate buffer, pH 6.0, 37°C 1 hr), mannosidase

(MANase I,·Glyko, 50 mM sodium phosphate, pH 6.0, 37°C 18 hr),

15   fucosidase (FUCase I, Glyko, 50 mM sodium phosphate, pH 5.0, 37°C 18

hr), N-acetylglucosaminidase (HEXase I, Glyko, 50 mM sodium phosphate,

pH 5.0, 37°C 4 hr), O-glycosidase (Glyko, 50 mM sodium phosphate, pH

5.0, 37°C 18 hr) or N-glycanase (PNGase F, Glyko, 100 mM sodium

phosphate, pH 8.6, 37°C, 18 hr).  The fragmented glycopeptides on the

20   surface are finally washed with phosphate buffered saline and water to

remove the reagents and reaction products.  An aliquot of 1 μL of sinapinic

acid is added to the surface and analyzed again by laser desorption time-of-

flight mass spectrometry.  Nascent sequences of the glycopeptides are

observed.  The sequences are deduced by the differences in the mass

25   between two peaks.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was

5      specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The oligonucleotides, compounds, methods, procedures and techniques

10     described herein are presently representative of the preferred embodiments, are intended to be exemplary and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims.

CLAIMS

What is claimed:

1.      An apparatus for measuring the mass of an analyte molecule

of an analyte sample by means of mass spectrometry, said apparatus

5      comprising:

a spectrometer tube;

vacuum means for applying a vacuum to the interior of said tube;

electrical potential means within the tube for applying an

accelerating electrical potential to desorbed analyte molecules from said

10      analyte sample;

sample presenting means removably insertable into said

spectrometer tube, for presenting said analyte sample in association with

surface associated molecule for promoting desorption and ionization of said

analyte molecules, wherein said surface molecule is selected from the group

15      consisting of energy absorbing molecule, affinity capture device, photolabile

attachment molecule and combination thereof;

an analyte sample deposited on said sample presenting means in

association with said surface associated molecules; whereby at least a

portion of said analyte molecules not consumed in said mass spectrometry

20      analysis will remain accessible for subsequent chemical, biological or

physical analytical procedures;

laser beam means for producing a laser beam directed to said

analyte sample for imparting sufficient energy to desorb and ionize a

portion of said analyte molecules from said analyte sample; and

detector means associated with said spectrometer tube for detecting the impact of accelerated ionized analyte molecules thereon.

2. A method in mass spectrometry to measure the mass of an analyte molecule, said method comprising the steps of:

5        derivitizing a sample presenting surface on a probe tip face with an affinity capture device having means for binding with an analyte molecule;

exposing said derivitized probe tip face to a source of said analyte molecule so as to bind said analyte molecule thereto;

placing the derivitized probe tip with said analyte molecules bound

10      thereto into one end of a time-of-flight mass spectrometer and applying a vacuum and an electric field to form an accelerating potential within the spectrometer;

striking at least a portion of the analyte molecules bound to said derivitized probe tip face within the spectrometer with one or more laser

15      pulses in order to desorb ions of said analyte molecules from said tip;

detecting the mass of the ions by their time of flight within said mass spectrometer; and

displaying such detected mass.

3. The method according to claim 2, further comprising applying

20      a desorption/ionization assisting matrix material to said probe tip face in association with said affinity capture device.

4. The method according to claim 3, further comprising removing said probe tip from said mass spectrometer;

performing a chemical or biological procedure on said portion of said analyte molecules not desorbed to alter the composition of said portion of said analyte molecules not desorbed;

reinserting said probe tip with said altered analyte molecules

5 thereon; and

performing subsequent mass spectrometry analysis to determine the molecular weight of said altered analyte molecules.

5. The method according to claim 2 wherein said affinity capture device is chemically bonded to said face of said probe tip.

10 6. The method according to claim 2 wherein said affinity capture device is physically adhered to said face of said probe tip.

7. The method according to claim 2 wherein said affinity capture device is adapted to chemically bond to said analyte molecules.

8. The method according to claim 2 wherein said affinity capture

15 device is adapted to biologically adhere to said analyte molecules.

9. The method according to claim 2 wherein said analyte molecules are biomolecules and said affinity reagent is adapted to selectively isolate said biomolecules from an undifferentiated biological sample.

20 10. The method according to claim 3 wherein said matrix materials are in the weakly acidic to strongly basic pH range.

11. The method according to claim 3 wherein said matrix materials have a pH above 6.0.

12. The method according to claim 2 wherein said face of said

25 probe tip is formed of an electrically insulating material.

13. A method of measuring the mass of analyte molecules by means of laser desorption/ionization, time-of-flight mass spectrometry in which an energy absorbing material is used in conjunction with said analyte molecules for facilitating desorption and ionization of the analyte

5          molecules, the improvement comprising:

presenting the analyte molecules on or above the surface of the energy absorbing material, wherein at least a portion of the analyte molecules not desorbed in said mass spectrometry analysis remain chemically accessible for subsequent analytical procedures.

10          14. An apparatus for facilitating desorption and ionization of analyte molecules, said apparatus comprising:

a sample presenting surface; and

surface associated molecules, wherein said surface associated molecules are selected from the group consisting of energy absorbing

15          molecule, affinity capture device, photolabile attachment molecule and combination thereof, said surface associated molecules associated with said sample presenting surface and having means for binding with said analyte molecules.

15. The apparatus according to claim 14 wherein said sample

20          presenting surface comprises the surface of a probe tip for use in a time-of-flight mass spectrometry analyzer.

16. The apparatus according to claim 14 wherein said affinity capture device or photolabile attachment molecule is chemically bonded to said sample presenting surface.

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17.     The apparatus according to claim 14 wherein said affinity capture device is physically adhered to said sample presenting surface.

18.     The apparatus according to claim 14 wherein said affinity capture device or photolabile attachment molecule is chemically bonded to said analyte molecules..

19.     The apparatus according to claim 14 wherein said affinity capture device is adapted to biologically adhere to said analyte molecules.

20.     The apparatus according to claim 14 wherein said analyte molecules are biomolecules and said affinity capture device or photolabile attachment molecule is adapted to selectively isolate said biomolecules from an undifferentiated biological sample.

21.     The apparatus according to claim 14, further comprising a matrix material deposited on said sample presenting surface in association with said affinity capture device or photolabile attachment molecule.

22.     The apparatus according to claim 21 wherein said matrix material is in the weakly acidic to strongly basic pH range.

23.     The apparatus according to claim 21 wherein said matrix material has a pH above 6.0.

24.     The apparatus according to claim 14 wherein said sample presenting surface is formed of an electrically insulating material.

25.     A method for capturing analyte molecules on a sample presenting surface and desorbing/ionizing said captured analyte molecules from said sample presenting surface for subsequent analysis, said method comprising:

derivitizing said sample presenting surface with an affinity capture device or photolabile attachment molecule having means for binding with said analyte molecules;

exposing said derivitized sample present surface to a sample

5      containing said analyte molecules;

capturing said analyte molecules on said derivitized sample presenting surface by means of said affinity capture device or photolabile attachment molecule; and

exposing said analyte molecules, while bound to said derivitized

10     sample presenting surface by means of said affinity capture device or photolabile attachment molecule, to an energy or light source to desorb at least a portion of said analyte molecules from said surface.

26.     A method for preparing a surface for presenting analyte molecules for analysis, said method comprising:

15         providing a substrate on said surface for supporting said analyte;

derivitizing said substrate with an affinity capture device or photolabile attachment molecule having means for selectively bonding with said analyte; and

a means for detecting said analyte molecules bonded with said

20     affinity capture device or photolabile attachment molecule.

27.     The method according to claim 26 comprising additionally the step of applying a detection material to said surface.

28.     The method according to claim 27 wherein such detection material comprises a fluorescing species.

29.    The method according to claim 27 wherein such detection material comprises an enzymatic species.

30.    The method according to claim 27 comprising additionally wherein such detection material comprises a radioactive species.

31.    The method according to claim 27 comprising additionally wherein such detection material comprises a light-emitting species.

32.    The method of claim 25, further comprising, depositing a desorption/ionization assisting material to said sample presenting surface in association with said affinity capture device or photolabile attachment molecule.

33.    The method of claim 25 wherein said energy source comprises a laser.

34.    The method of claim 25 wherein an affinity capture device is used and said energy source comprises an ion source.

35.    The method of claim 25 wherein a portion of said analyte molecules remain bound to said sample presenting surface after exposure to said energy source.

36.    The method of claim 35, further comprising the steps of:

converting at least a portion of the analyte molecules remaining bound on said derivitized sample presenting surface to modified analyte molecules by a chemical, biological or physical reaction, wherein said analyte molecules remain bound to said derivitized sample presenting surface by means of said affinity capture device or photolabile attachment molecule; and

exposing said modified analyte molecules to an energy source so as

to desorb at least a portion of said modified analyte molecules from said

surface.

37.     A sample probe for promoting desorption of intact analytes

5       into the gas phase comprising:

a sample presenting surface; and

an energy absorbing molecule associated with said sample

presenting surface, wherein said sample probe promotes desorption of an

intact analyte molecule positioned on, above or between the energy

10      absorbing molecules when said sample probe is impinged by an energy

source.

38.     The sample probe of claim 37, wherein the energy absorbing

molecule is selected from the group consisting of cinnamamide, cinnamyl

bromide, 2, 5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid.

15           39.     The sample probe of claim 37, wherein the sample presenting

surface is selected from the group consisting of glass, ceramics, teflon

coated magnetic materials; organic polymers and native biopolymers.

40.     A sample probe for promoting desorption of intact analytes

into the gas phase comprising:

20                       a sample presenting surface; and

an affinity capture device associated with said sample

presenting surface; wherein, when said sample probe is impinged by

an energy source, said sample probe promotes the transition of an

intact analyte molecule into the gas phase.

41.    The sample probe of claim 40, wherein the affinity capture device is selected from the group consisting of metal ions, proteins, peptides, immunoglobulins, nucleic acids, carbohydrates, lectins, dyes, reducing agents and combination thereof.

5      42.    The sample probe of claim 40, wherein the sample presenting surface is selected from the group consisting of glass, ceramics, teflon coated magnetic materials; organic polymers and native biopolymers.

43.    A sample probe for promoting desorption of intact analytes into the gas phase comprising:

10              a sample presentation surface; and

a mixture of at least two different molecules selected from the group consisting of an affinity capture device, an energy absorbing molecule and a photolabile attachment molecule associated with said sample presentation surface; wherein when an analyte is associated

15     with said sample probe, said sample probe promotes the transition of the analyte into the gas phase when said sample probe is impinged by an energy source.

44.    The sample of claim 43, wherein the analyte is selectively desorbed from the mixture after impingement by the energy source.

20     45.    A sample probe for promoting of differential desorption of intact analyte into the gas phase, comprising:

a sample presentation surface; and

at least two different affinity capture devices associated with said sample presentation surface; wherein, when said sample probe

25     is impinged by an energy source, said sample probe promotes the

transition of an analyte molecule into the gas phase at different

rates depending on the affinity capture device associated with said

analyte molecule.

46.     The sample probe of claim 45, wherein the affinity devices are

5       arranged in predetermined arrays.

47.     The sample probe of claim 46, wherein the arrays selectively

absorb a plurality of different analytes.

48.     The apparatus with the sample probe of claim 40 for

quantitating an analyte, wherein the position and quantity of affinity

10      capture devices determines the quantity of analyte absorbed.

49.     The apparatus according to claim 2, 14 or 21, wherein the

binding is selective.

50.     The apparatus according to claim 2, 14 or 21, wherein the

binding is non-selective.

15      51.     The method according to claim 3, 4 or 25, wherein the

binding is selective.

52.     The method according to claim 3, 4 or 25, wherein the

binding is non-selective.

53.     The sample probe according to claim 40, 41, 43 or 45, wherein

20      the binding is non-selective.

54.     The sample probe according to claim 40, 41, 43 or 45, wherein

the binding is selective.

55.     A sample probe for promoting desorption of intact analyte

into the gas phase, comprising:

25              a sample presentation surface; and

a surface associated molecule, wherein said surface associated
molecule can function both as an energy absorbing molecule and as an
affinity capture device.

56.     A sample probe for desorption of intact analyte into the gas

5       phase, comprising:

a sample presentation surface; and

a surface associated molecule wherein said surface associated
molecule is a photolabile attachment molecule having at least two
binding sites, wherein at least one site is bound to the sample

10      presentation surface and at least one site is available to bind an
analyte and wherein the analyte binding site is photolabile.

57.     A method in mass spectrometry to measure the mass of an
analyte molecule, said method comprising the steps of:

derivitizing a sample presenting surface on a probe tip face with a

15      photolabile attachment molecule (PAM), wherein said PAM has at least
two binding sites, one binding site binds to the sample presenting surface
and at least one binding site is available for binding with an analyte
molecule;

exposing said derivitized probe tip face to a source of said analyte

20      molecule so as to bind said analyte molecule thereto;

placing the derivitized probe tip with said analyte molecules bound
thereto into one end of a time-of-flight mass spectrometer and applying a
vacuum and an electric field to form an accelerating potential within the
spectrometer;

striking at least a portion of the analyte molecules bound to said

derivitized probe tip face within the spectrometer with one or more laser

pulses in order to desorb ions of said analyte molecules from said tip;

detecting the mass of the ions by their time of flight within said

5    mass spectrometer; and

displaying such detected mass.

58.    The method according to claim 57, further comprising

applying a desorption/ionization assisting matrix material to said probe tip

face in association with said PAM.

10    59.    The method according to claim 58, further comprising

removing said probe tip from said mass spectrometer;

performing a chemical, biological or physical procedure on said

portion of said analyte molecules not desorbed to alter the composition of

said portion of said analyte molecules not desorbed;

15    reinserting said probe tip with said altered analyte molecules

thereon; and

performing subsequent mass spectrometry analysis to determine the

molecular weight of said altered analyte molecules.

60.    The method according to claim 57 wherein said PAM is

20    chemically bonded to said face of said probe tip.

61.    The method according to claim 57 wherein said PAM is

chemically bonded to said analyte molecule, and wherein said bond

between the PAM and the analyte molecule is broken and the analyte

molecule is released in a light dependent manner.

62.    The method according to claim 57 wherein said analyte

molecules are biomolecules and said PAM is adapted to selectively isolate

said biomolecules from an undifferentiated biological sample.

63.    The method according to claim 58 wherein said matrix

5      materials are in the weakly acidic to strongly basic pH range.

64.    The method according to claim 58 wherein said matrix

materials have a pH above 6.0.

65.    The method according to claim 57 wherein said face of said

probe tip is formed of an electrically insulating material.

10     66.    A method of measuring the mass of analyte molecules by

means of laser desorption/ionization, time-of-flight mass spectrometry in

which a photolabile attachment molecule (PAM) is used in conjunction

with said analyte molecules for facilitating desorption and ionization of the

analyte molecules, the improvement comprising:

15         presenting the analyte molecules on or above the surface of the

PAM, wherein at least a portion of the analyte molecules not desorbed in

said mass spectrometry analysis remain chemically accessible for

subsequent analytical procedures.

67.    A sample probe for promoting of differential desorption of

20     intact analyte into the gas phase, comprising:

a sample presentation surface; and

at least two different photolabile attachment molecules

associated with said sample presentation surface; wherein, when

said sample probe is impinged by an energy source, said sample

25         probe promotes the transition of an analyte molecule into the gas

phase at different rates depending on the photolabile attachment molecule associated with said analyte molecule.

68.     The sample probe of claim 67, wherein the photolabile attachment molecules are arranged in predetermined arrays.

5          69.     The sample probe of claim 68, wherein the arrays selectively absorb a plurality of different analytes.

70.     A sample probe for promoting desorption of intact analytes into the gas phase comprising:

a sample presenting surface; and

10               a photolabile attachment molecule associated with said sample presenting surface; wherein, when said sample probe is impinged by an energy source, said sample probe promotes the transition of an intact analyte molecule into the gas phase.

71.     The apparatus of claim 70 for quantitating an analyte,

15     wherein the position and quantity of photolabile attachment molecule determines the quantity of analyte absorbed.

72.     A method for biopolymer sequence determination comprising the steps of:

binding a biopolymer analyte to probe tip containing a sample

20     presenting surface having a surface selected molecule selected from the group consisting of an energy absorbing molecule, an affinity capture device, a photolabile attachment molecule and a combination thereof;

desorption of biopolymer analyte in mass spectrometry

analysis, wherein at least a portion of said biopolymer is not

desorbed from the probe tip;

analyzing the results of the desorption modifying the

5       biopolymer analyte still bound to the probe tip; and

repeating the desorption, analyzing and modifying steps until

the biopolymer is sequenced.

73.     The method of claim 72, wherein the biopolymer is selected

from the group consisting of protein, RNA, DNA and carbohydrate.

FIGURE 1

1 / 42
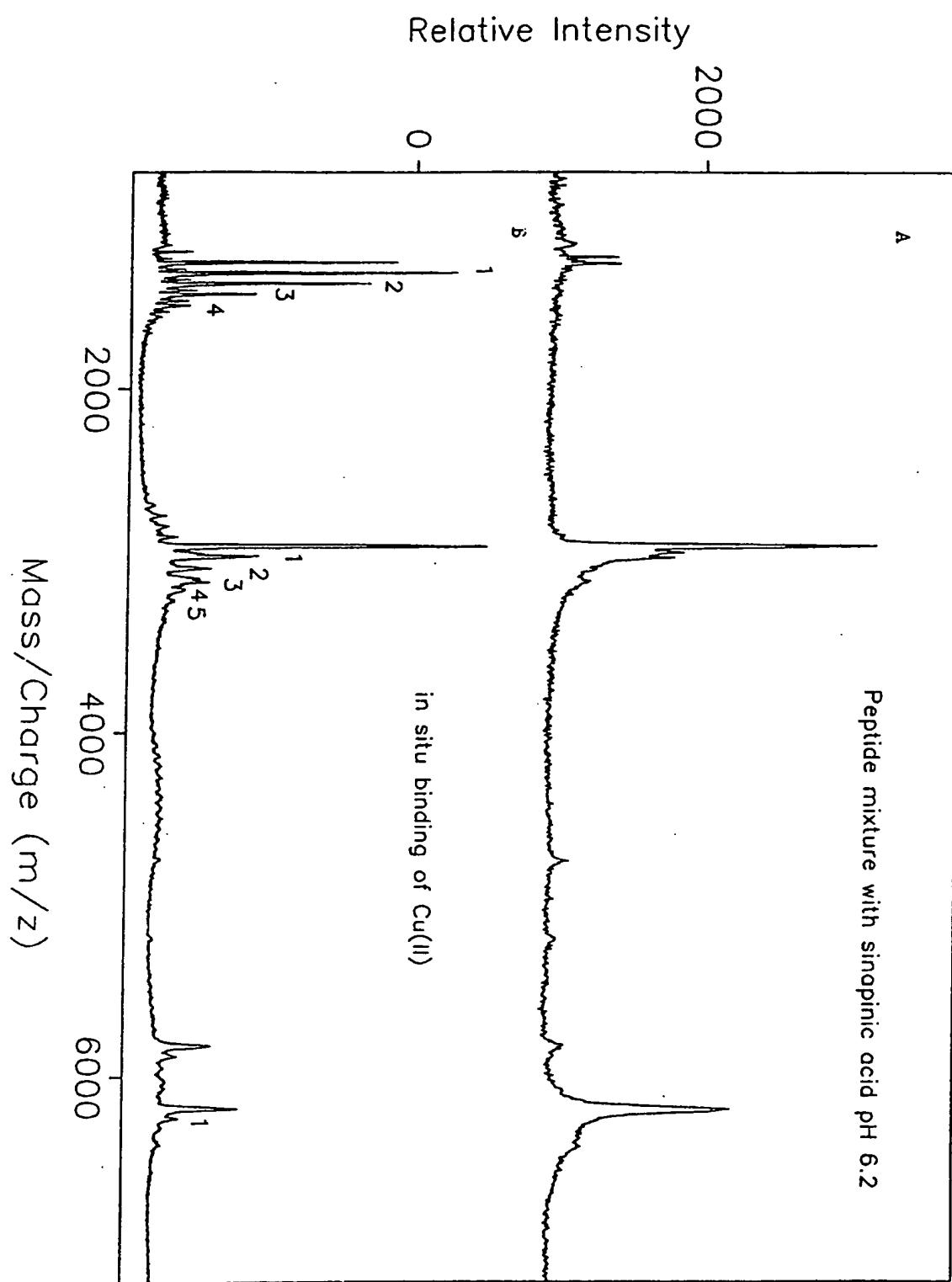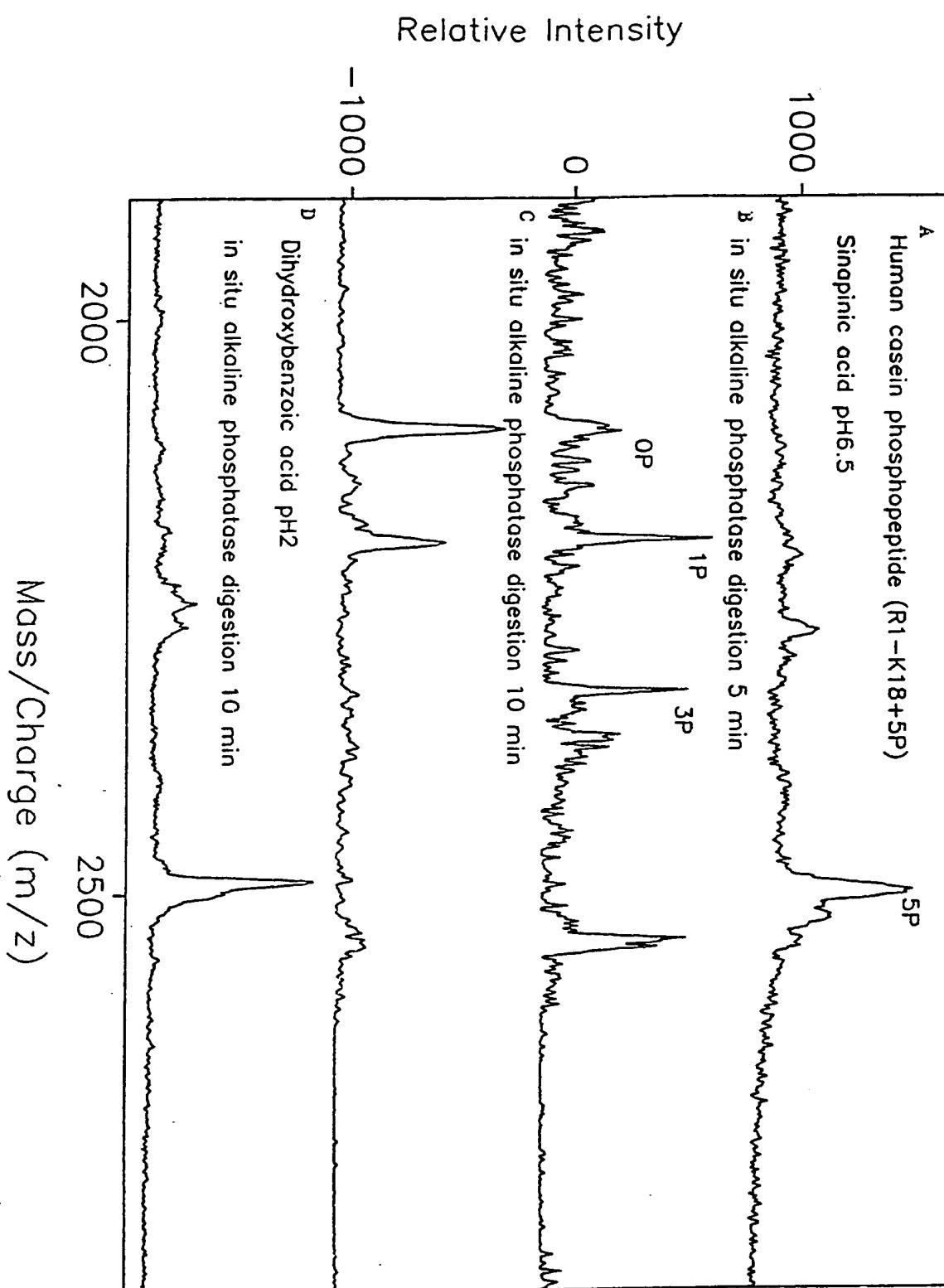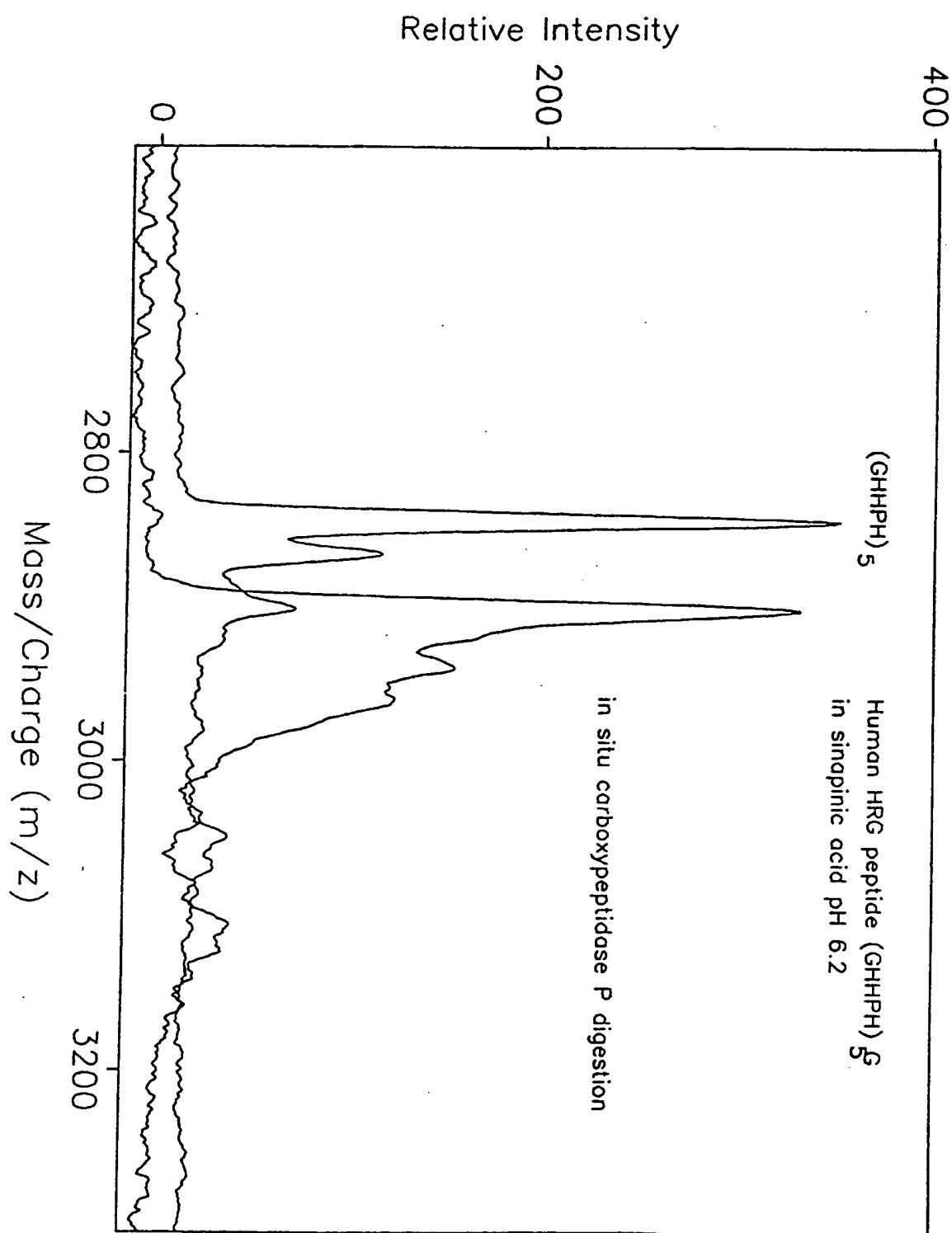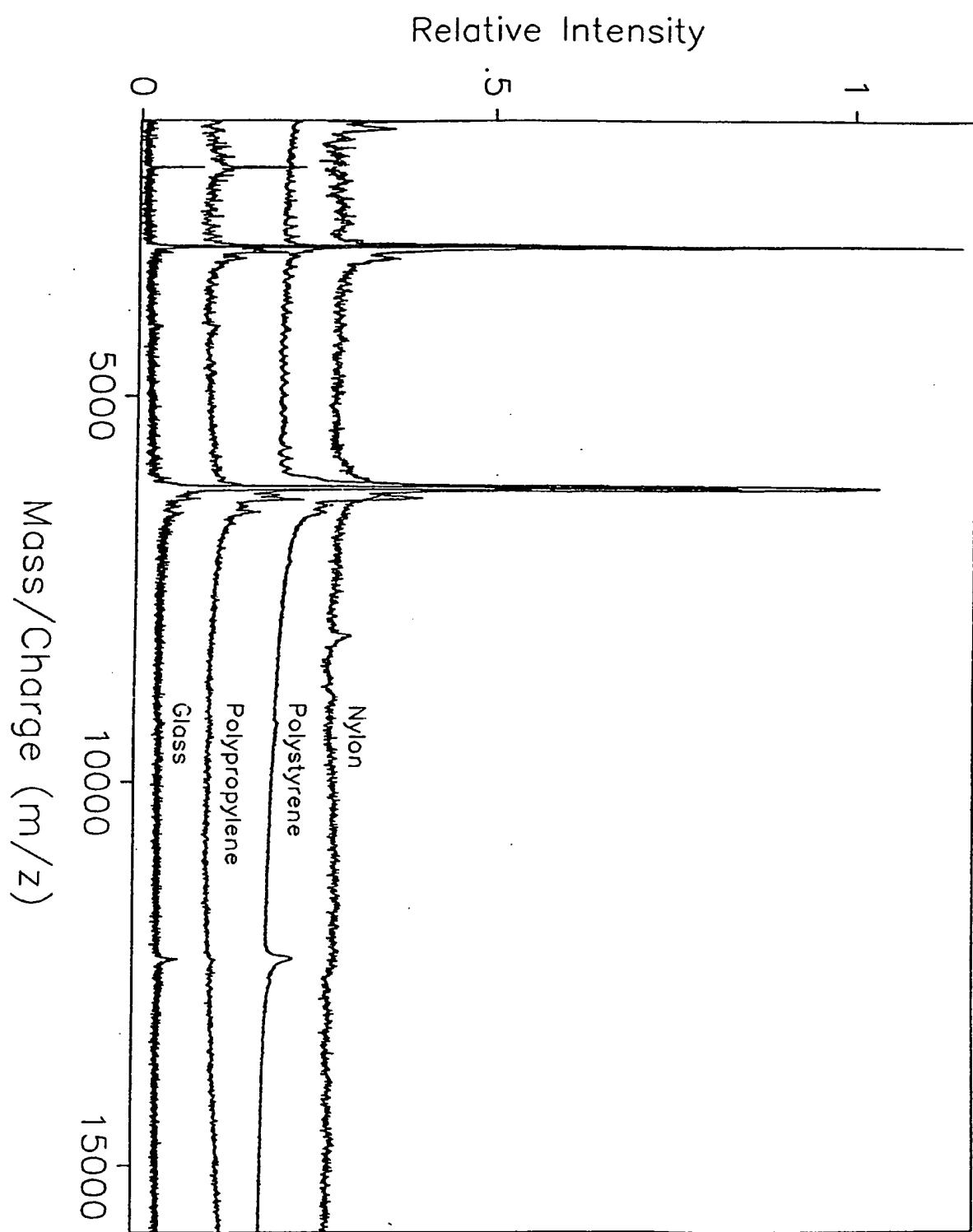
FIGURE 2

FIGURE 3

FIGURE 4
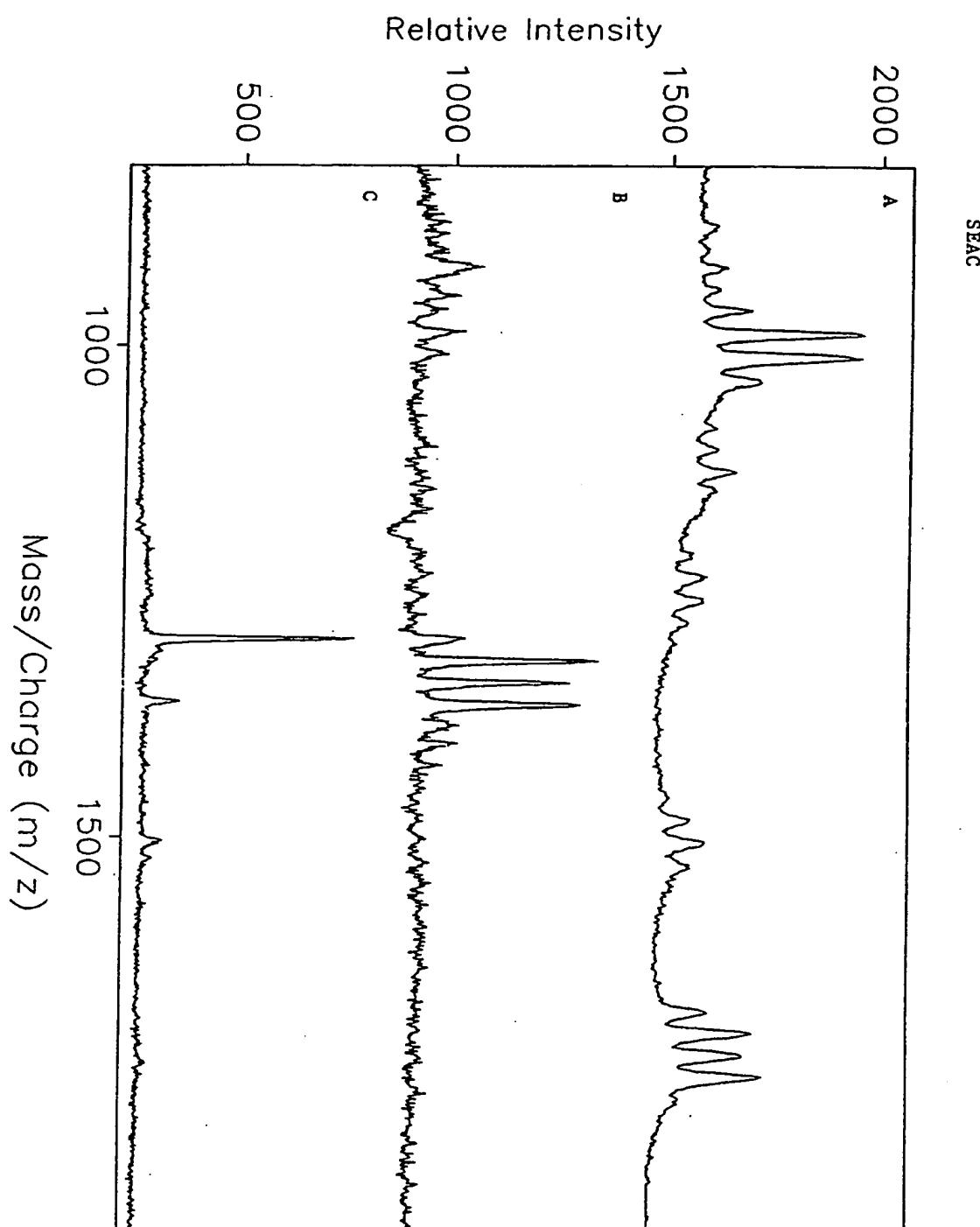
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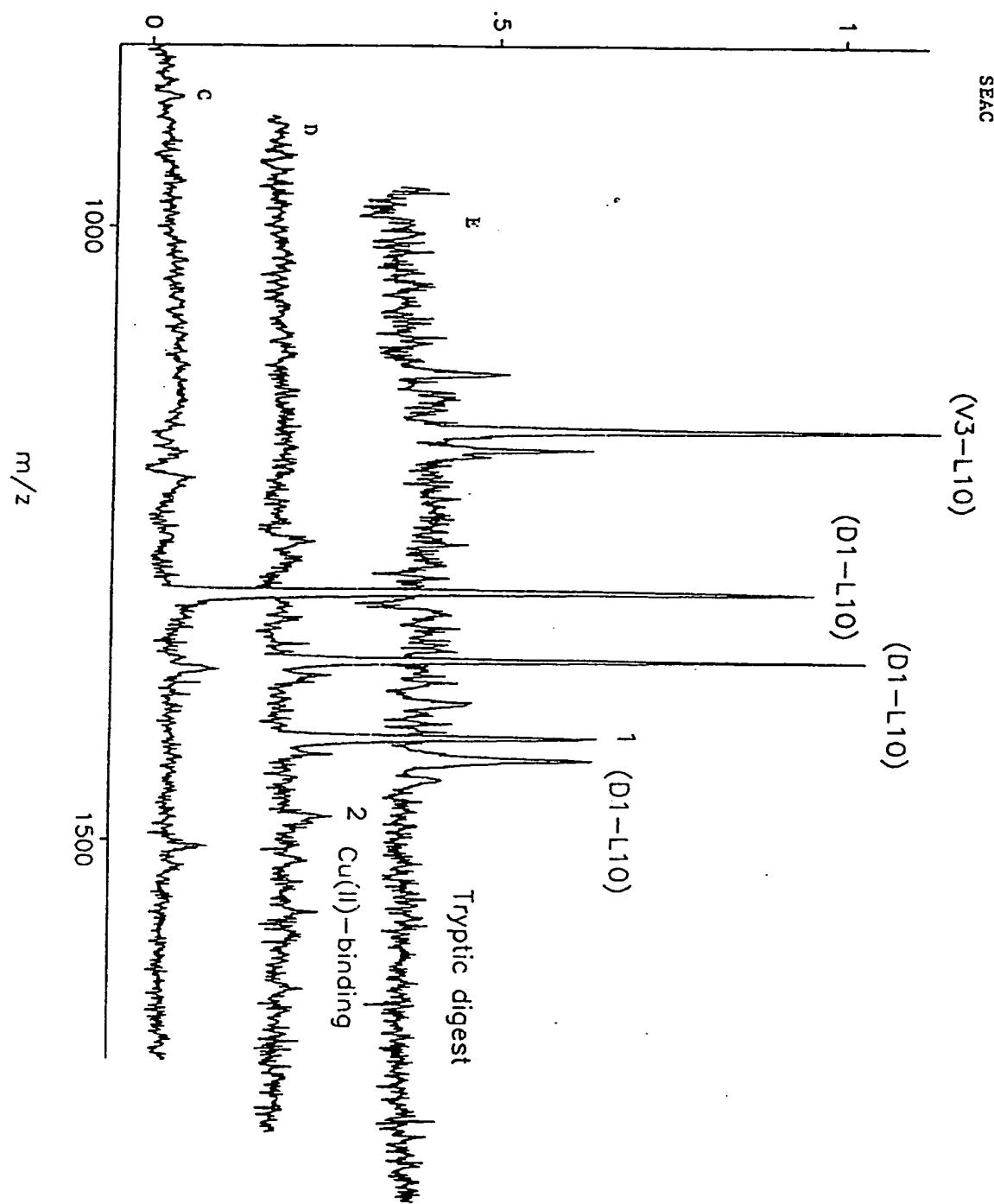
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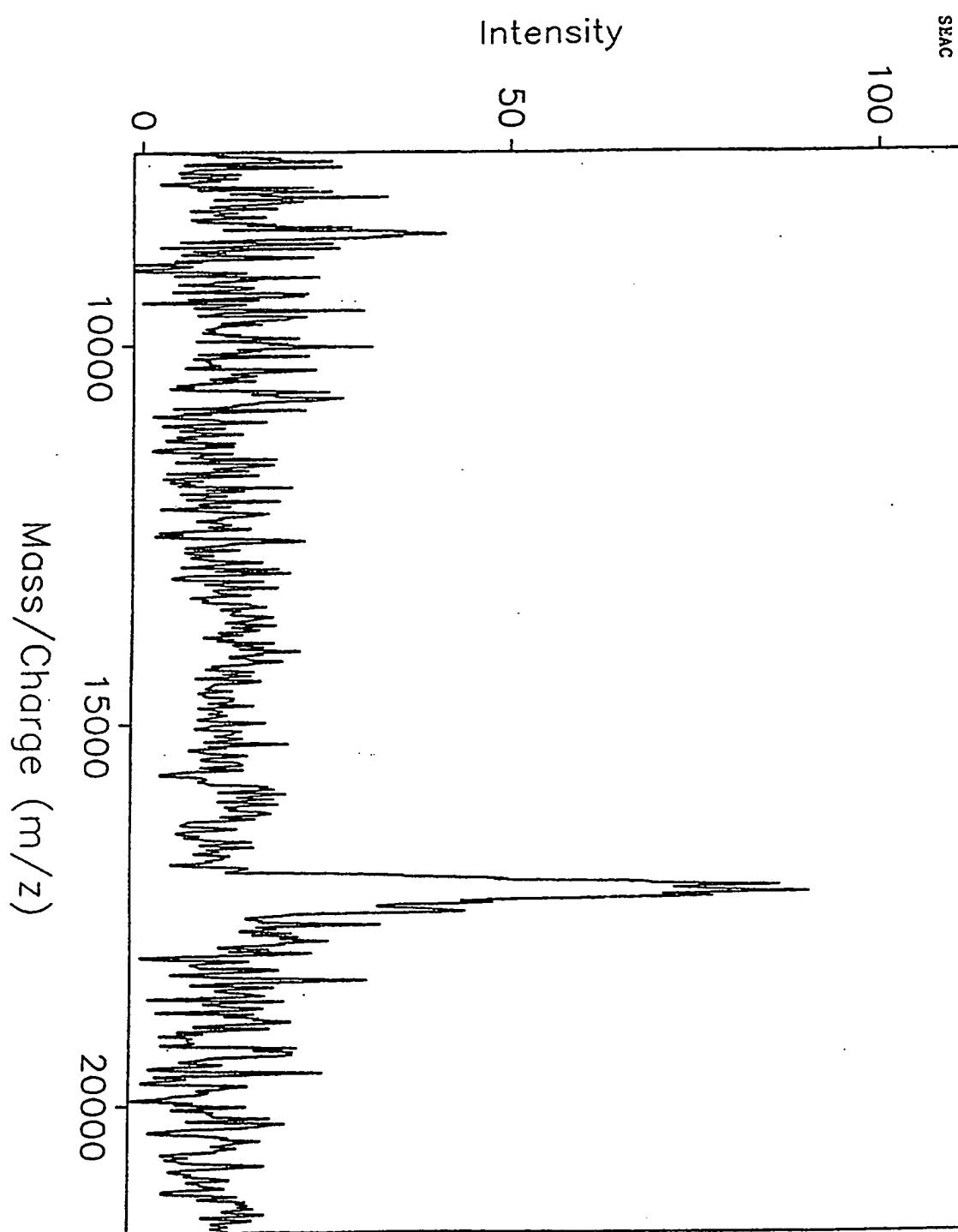
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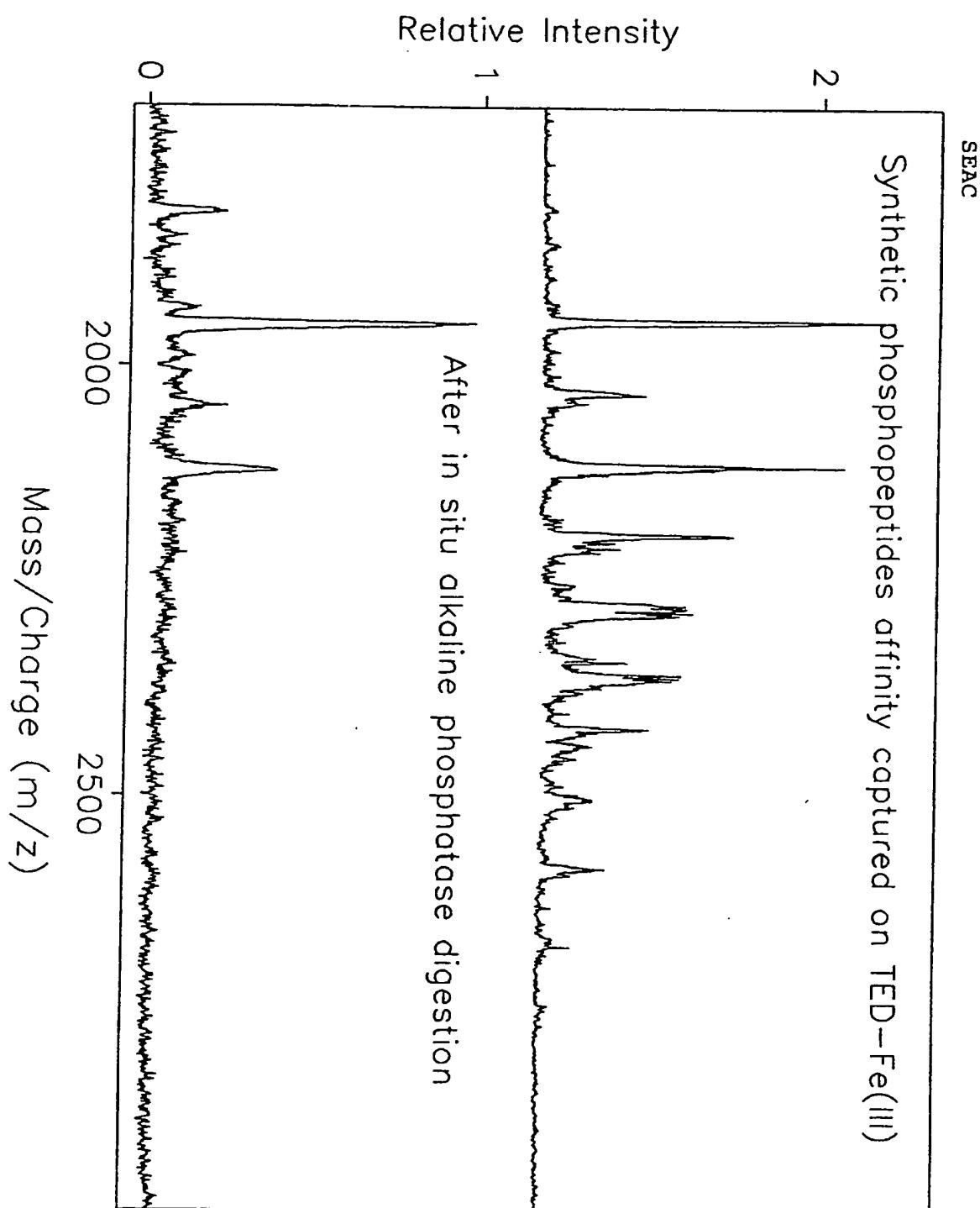
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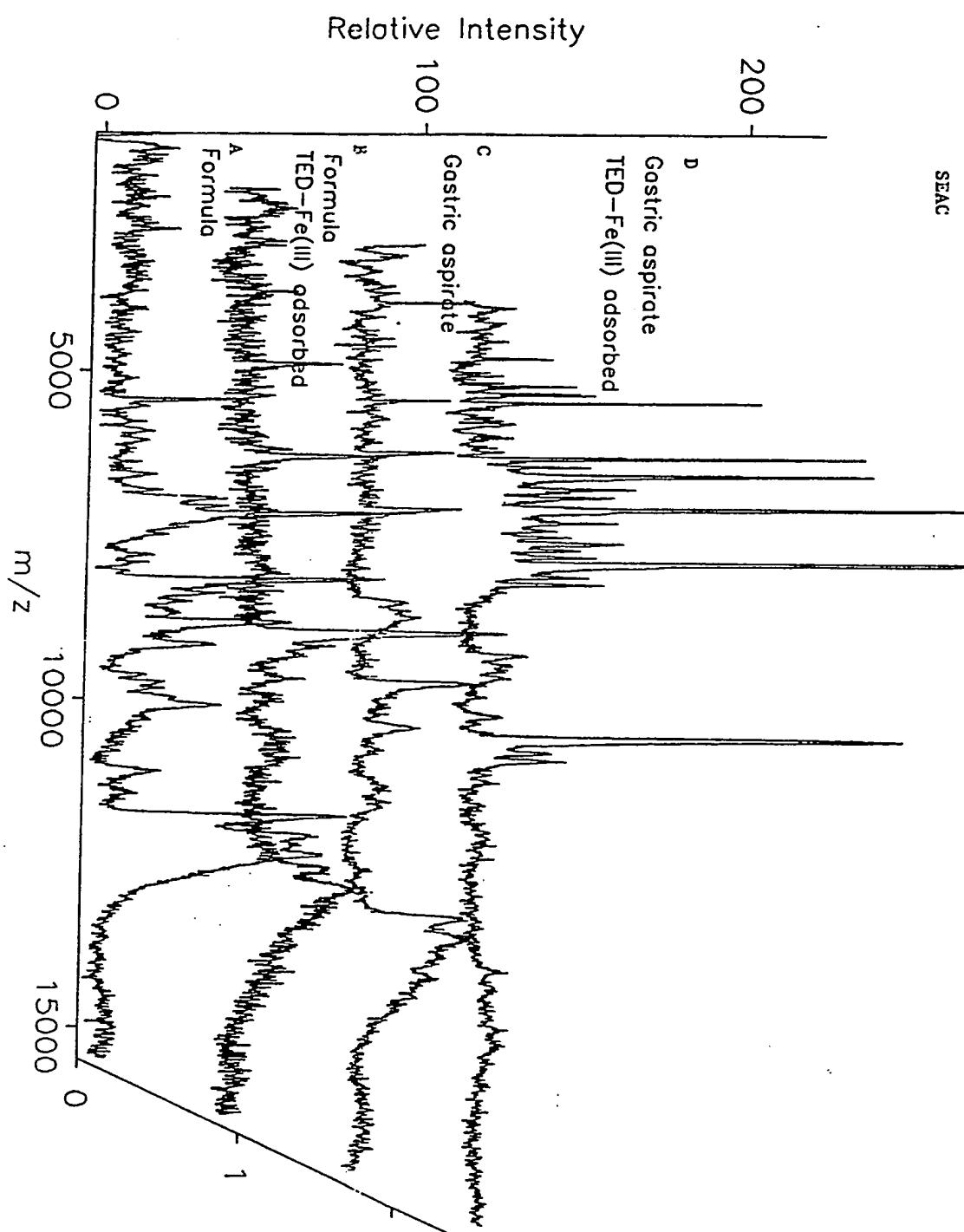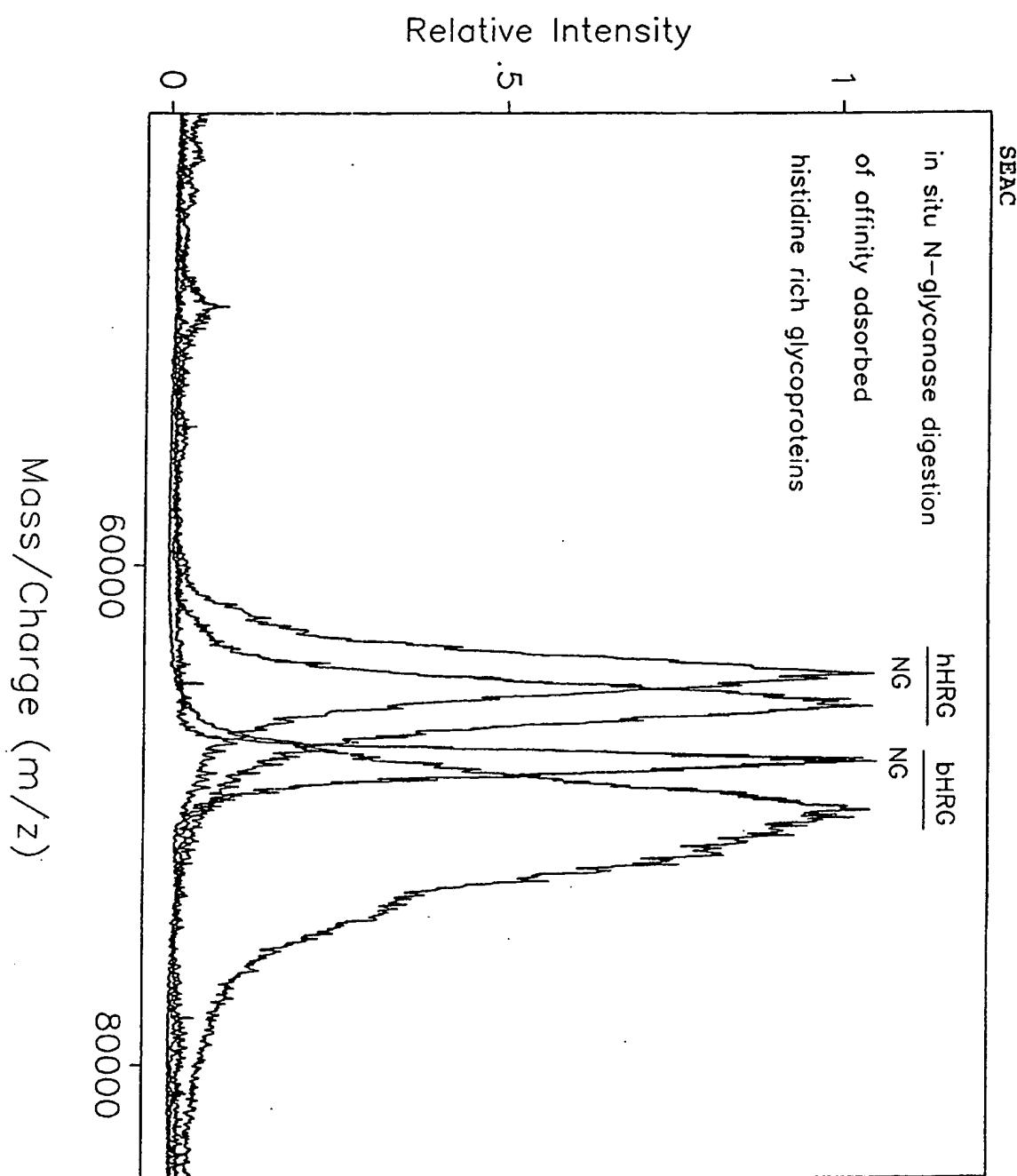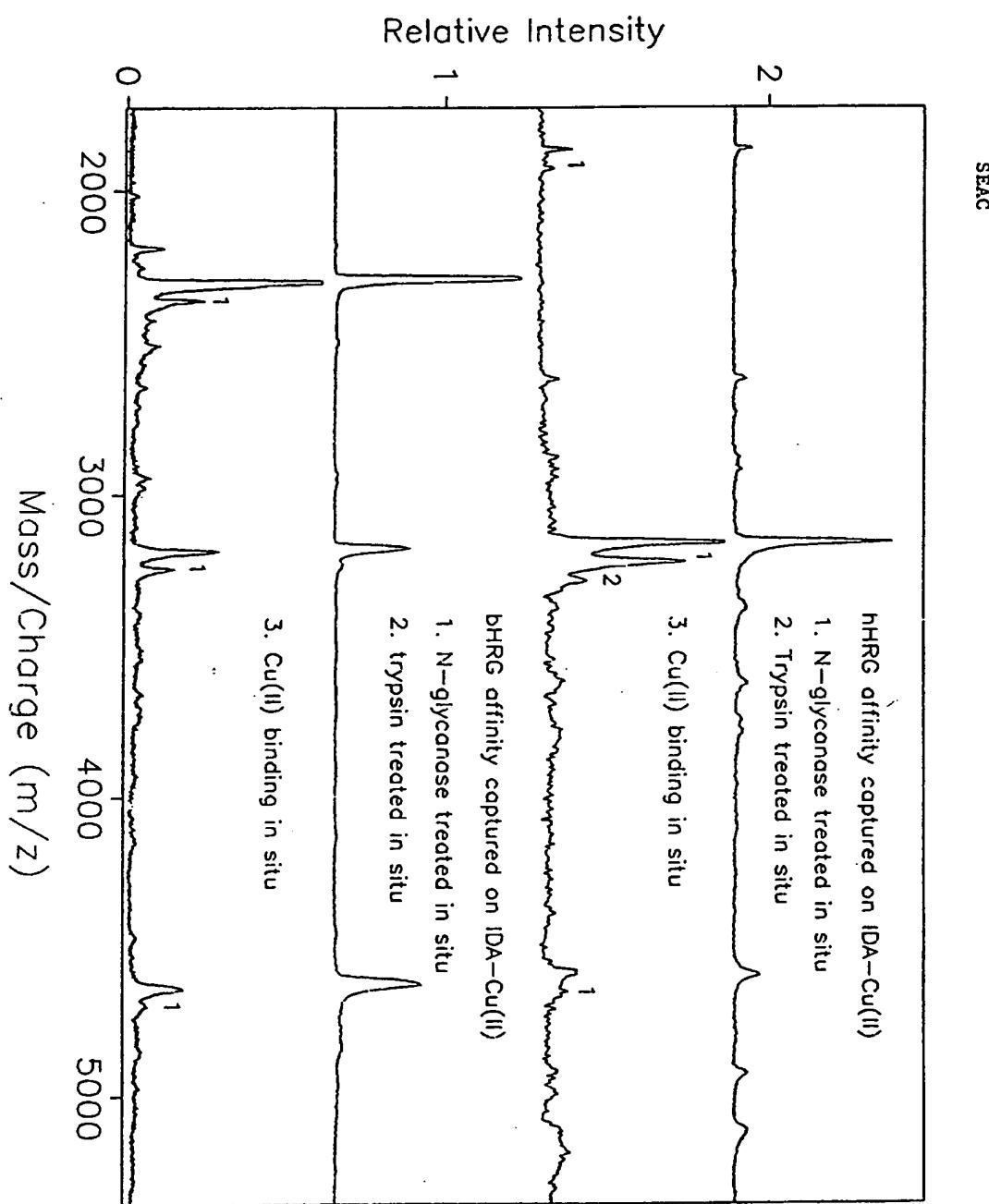
FIGURE 8

FIGURE 9A

FIGURE 9B
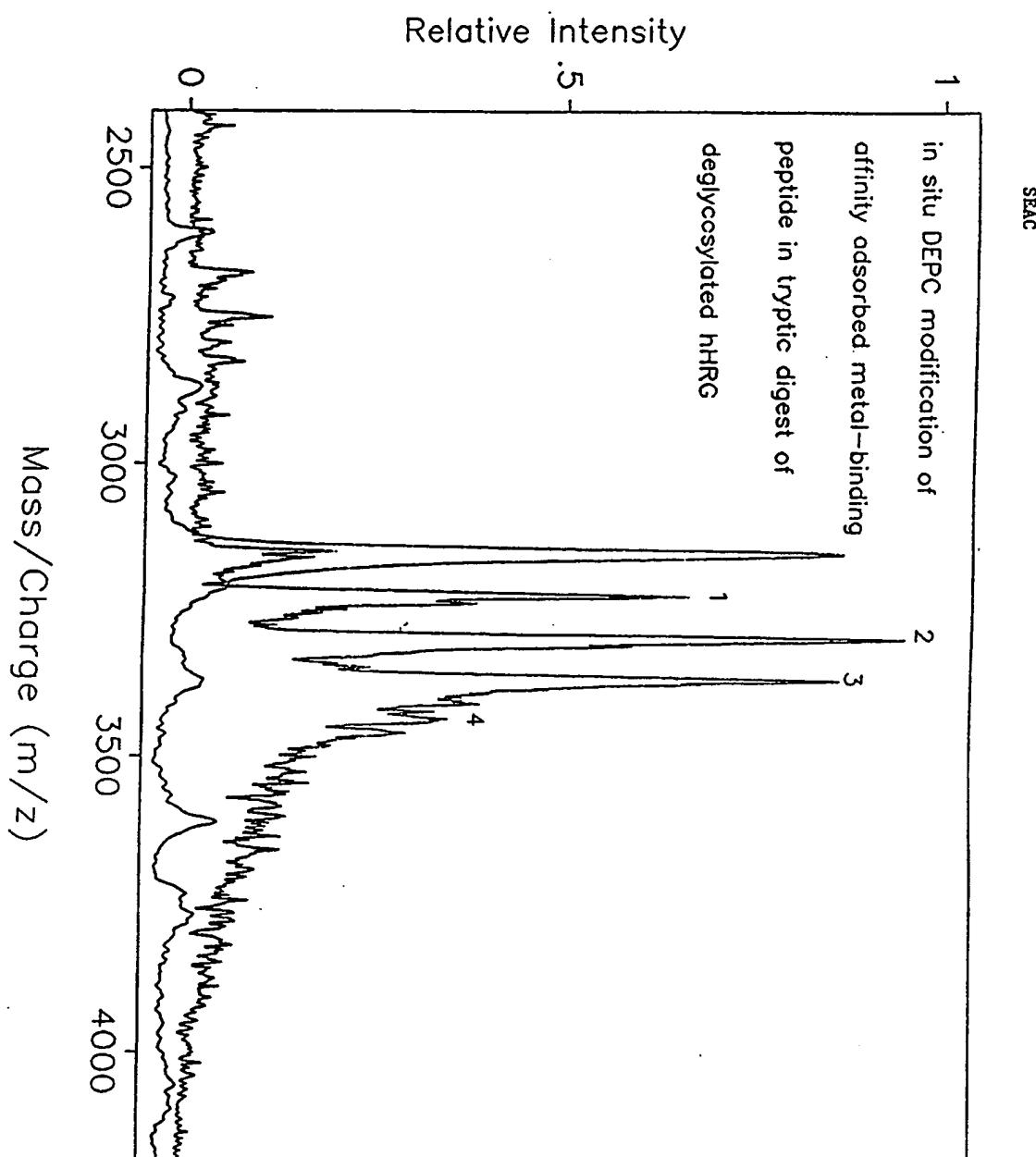
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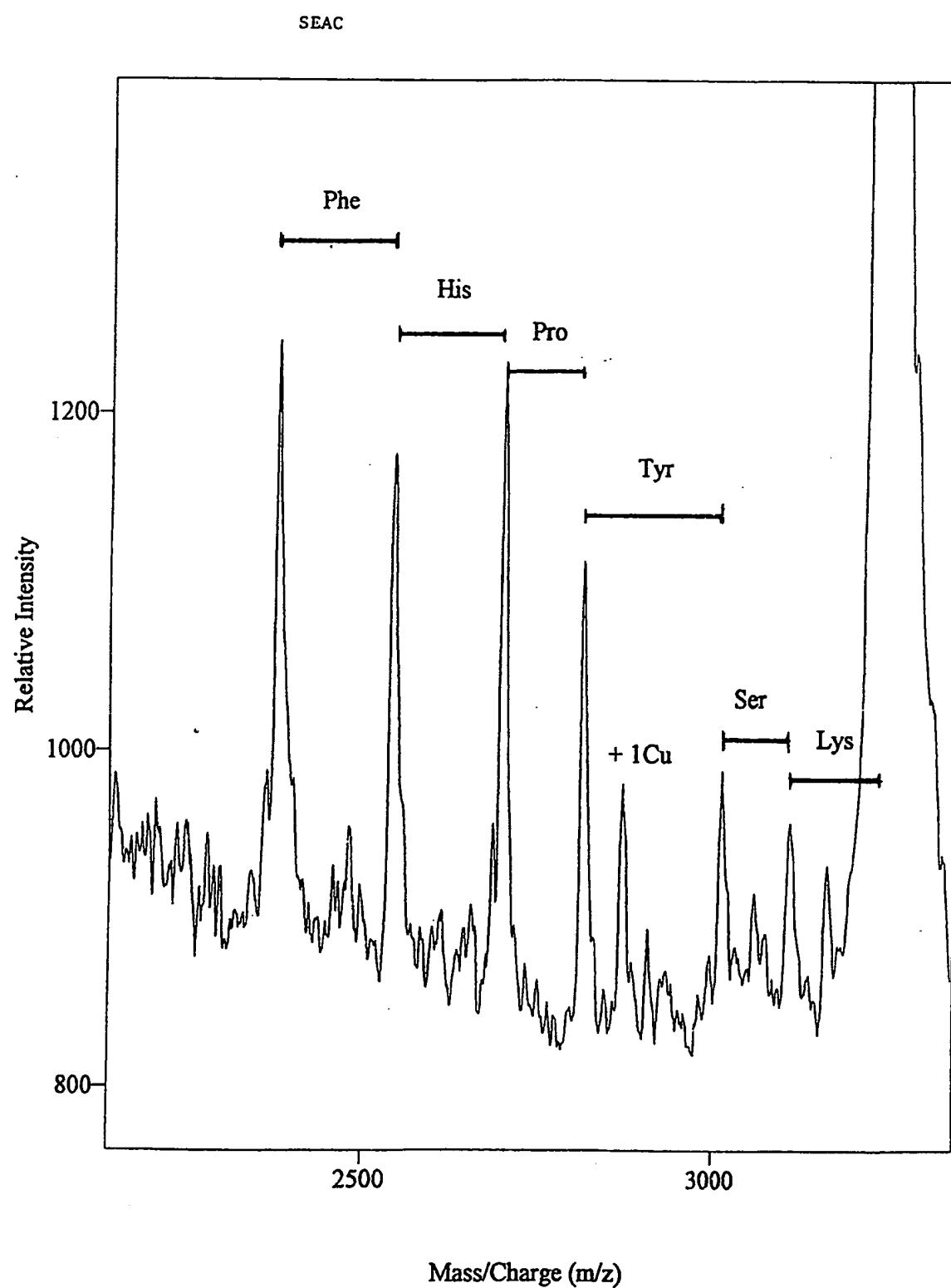
SEAC



FIGURE 9D

FIGURE 10

FIGURE 11

15 / 42
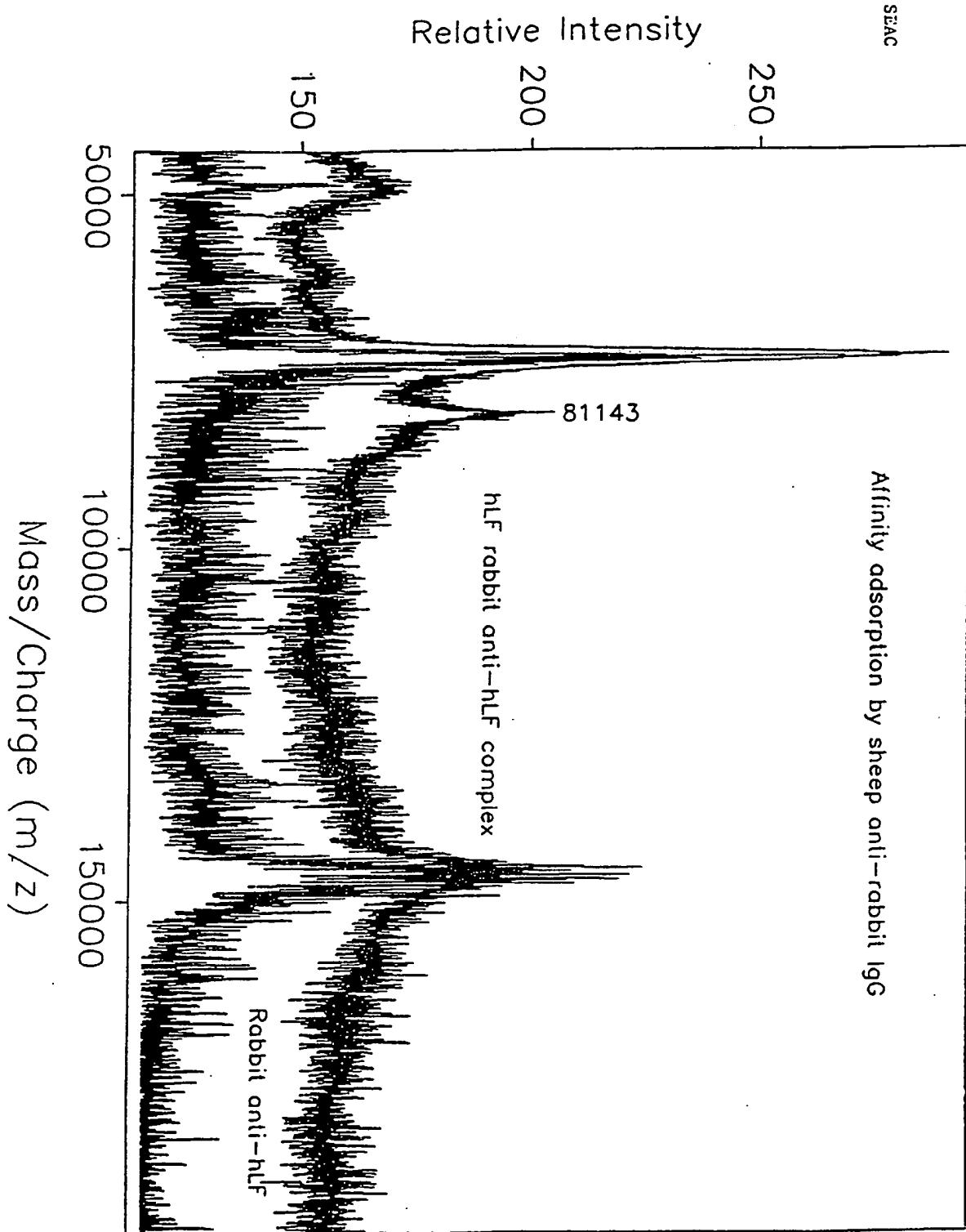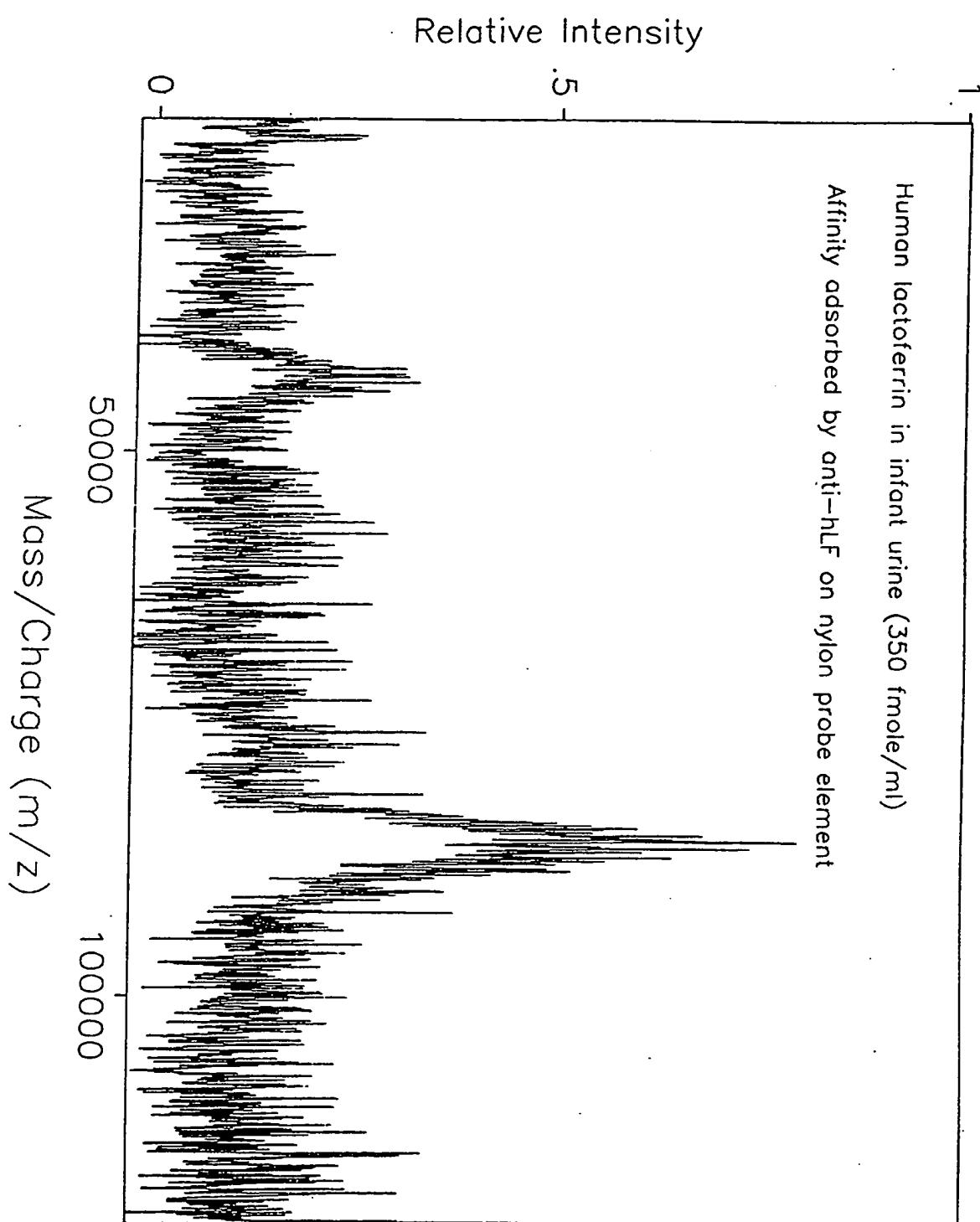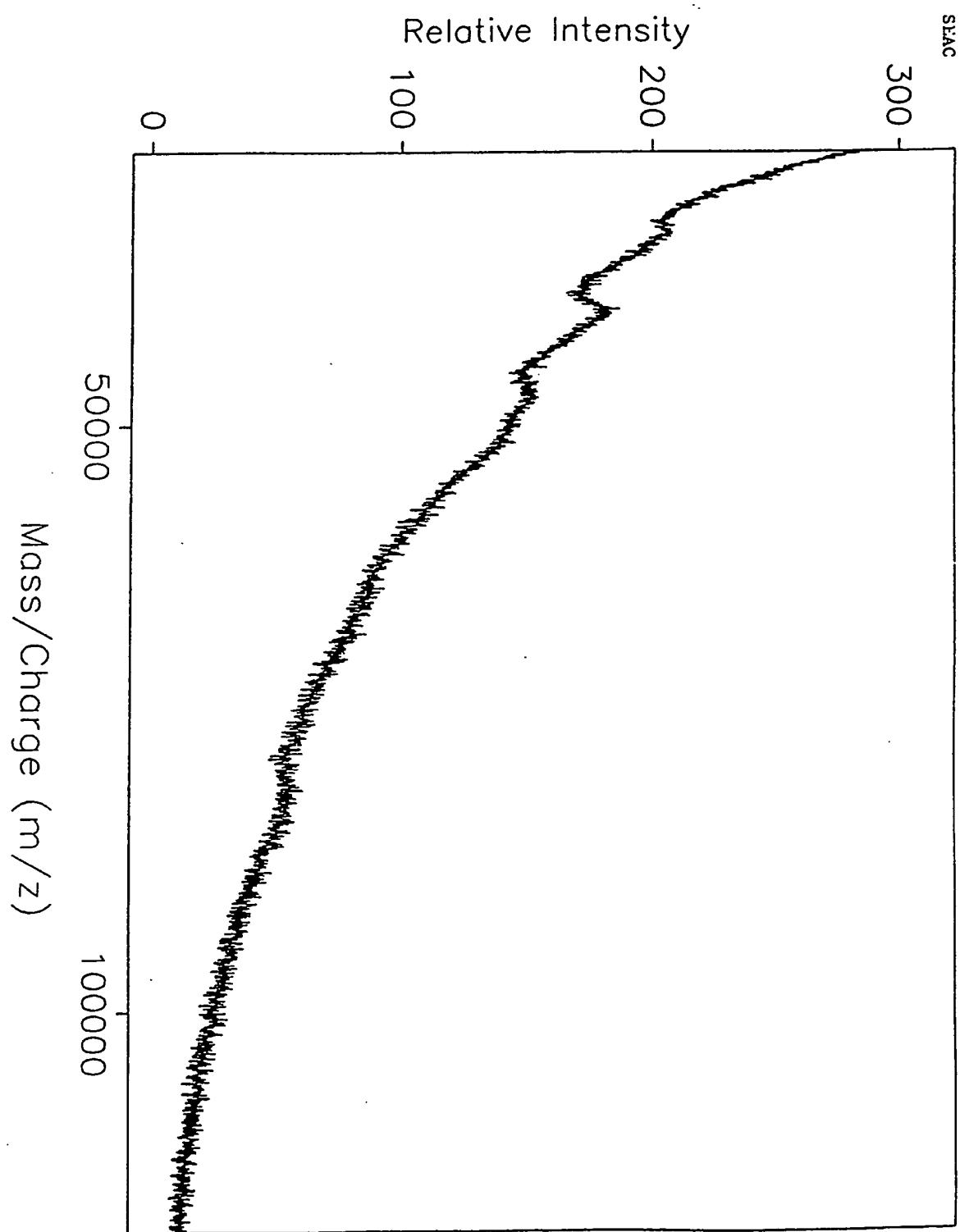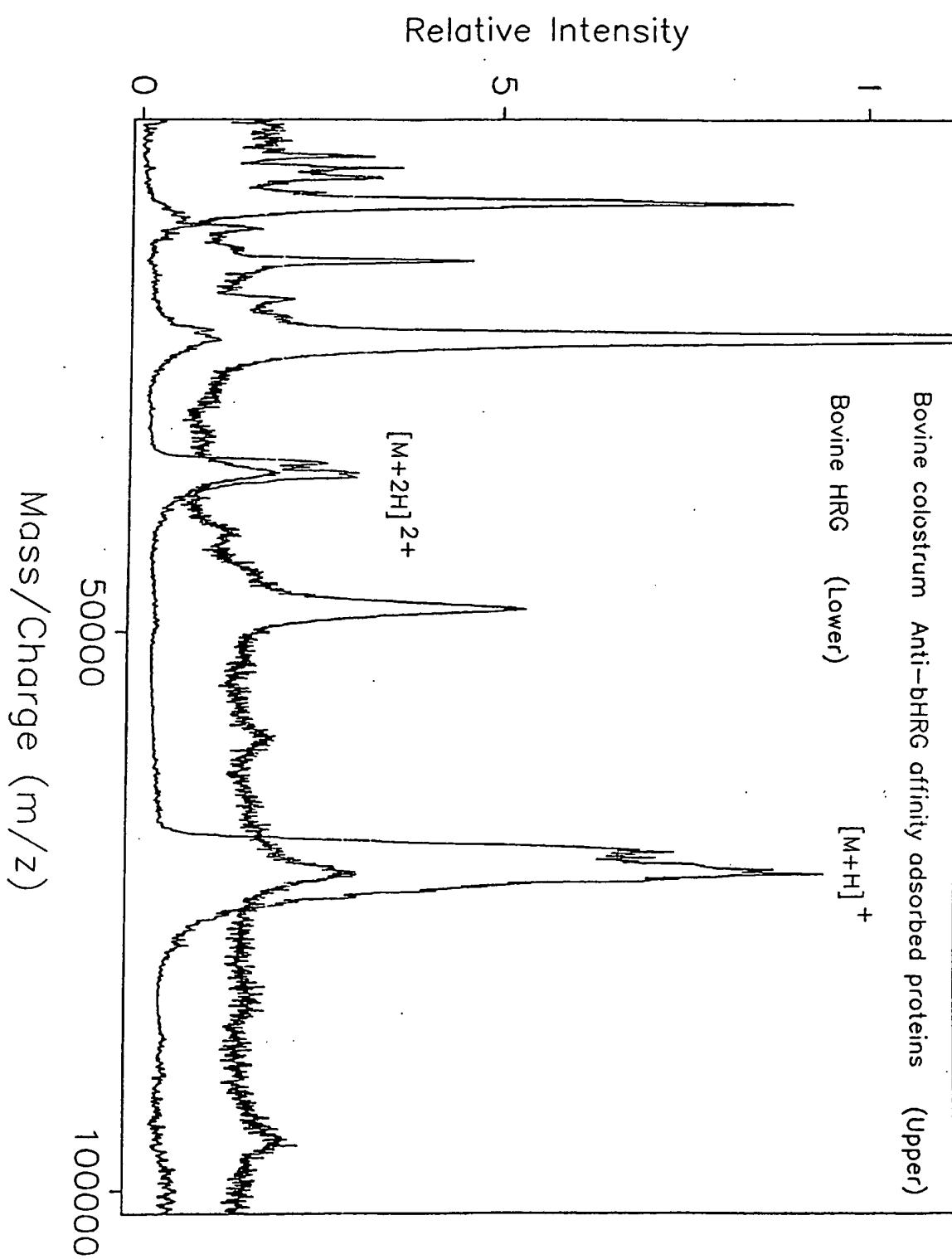
FIGURE 11

FIGURE 12

FIGURE 13

18 / 42
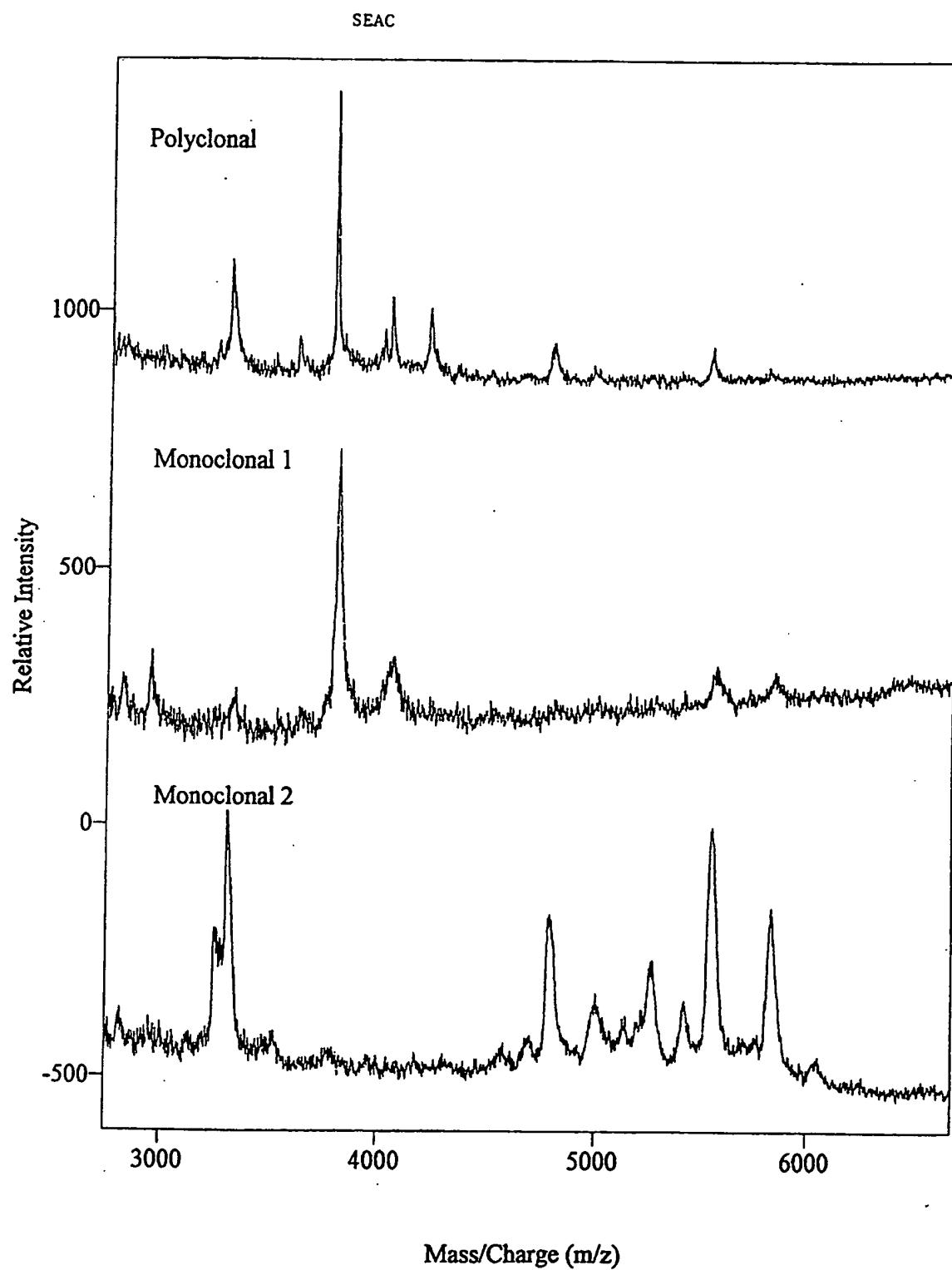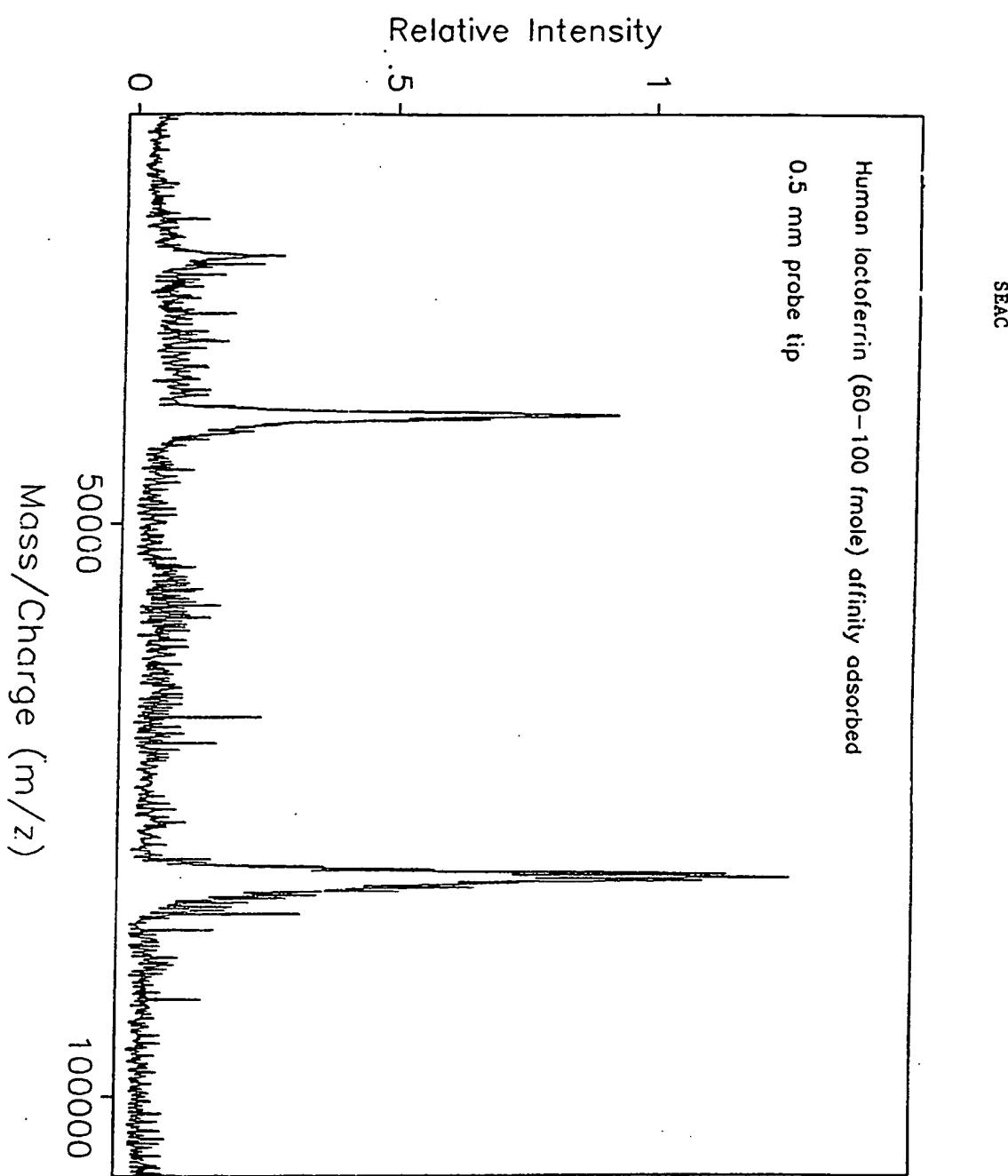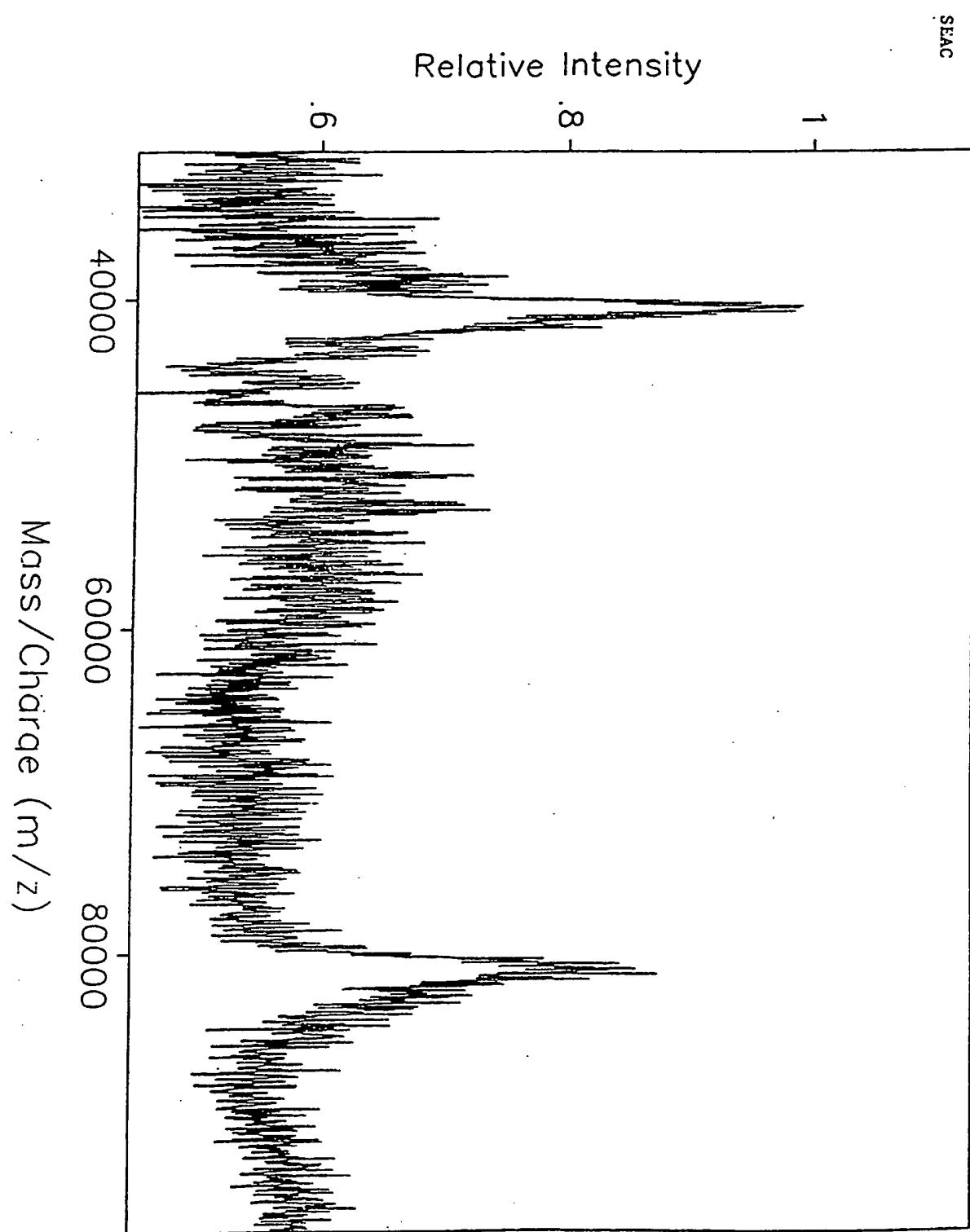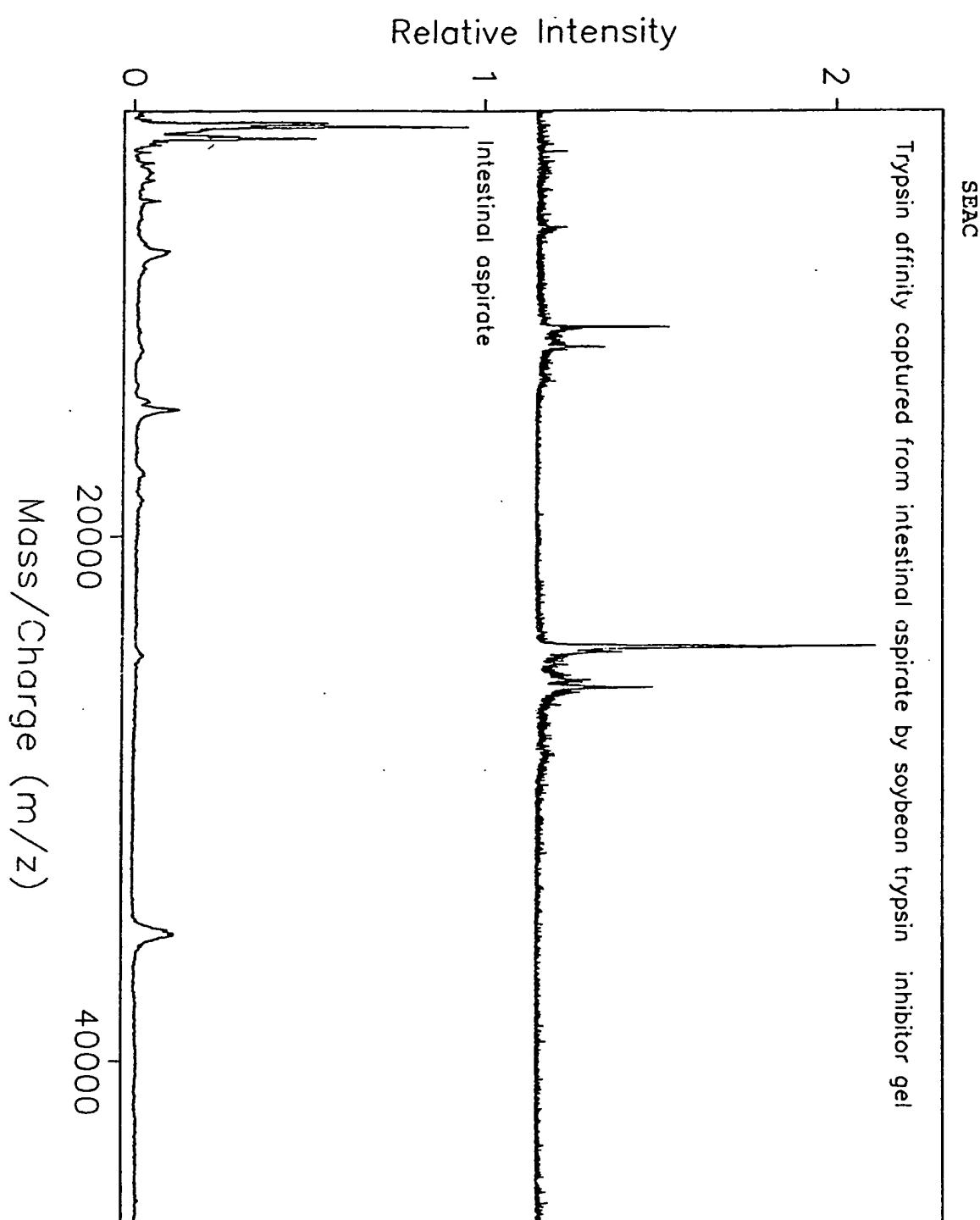
FIGURE 14

FIGURE 15

FIGURE 16A
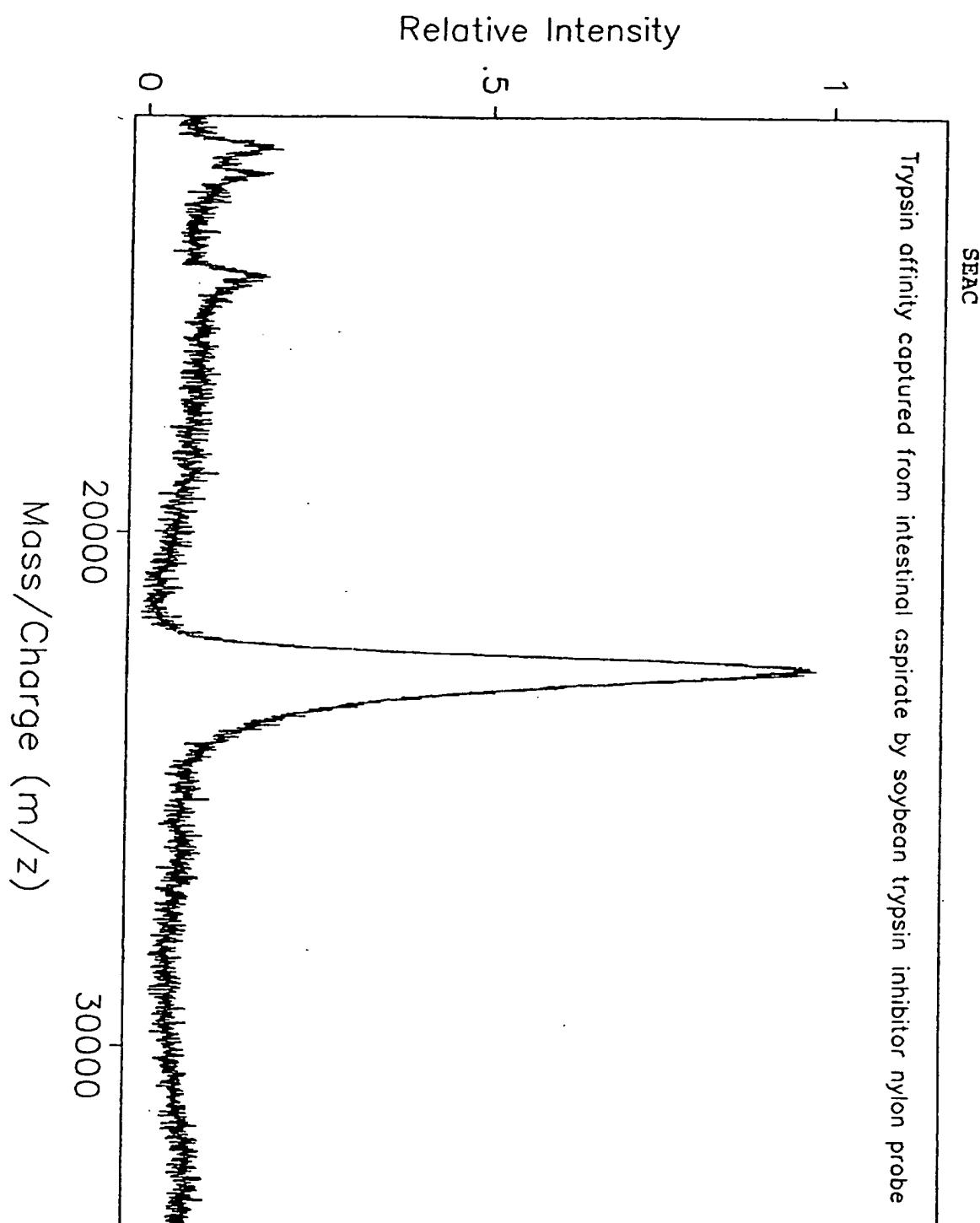
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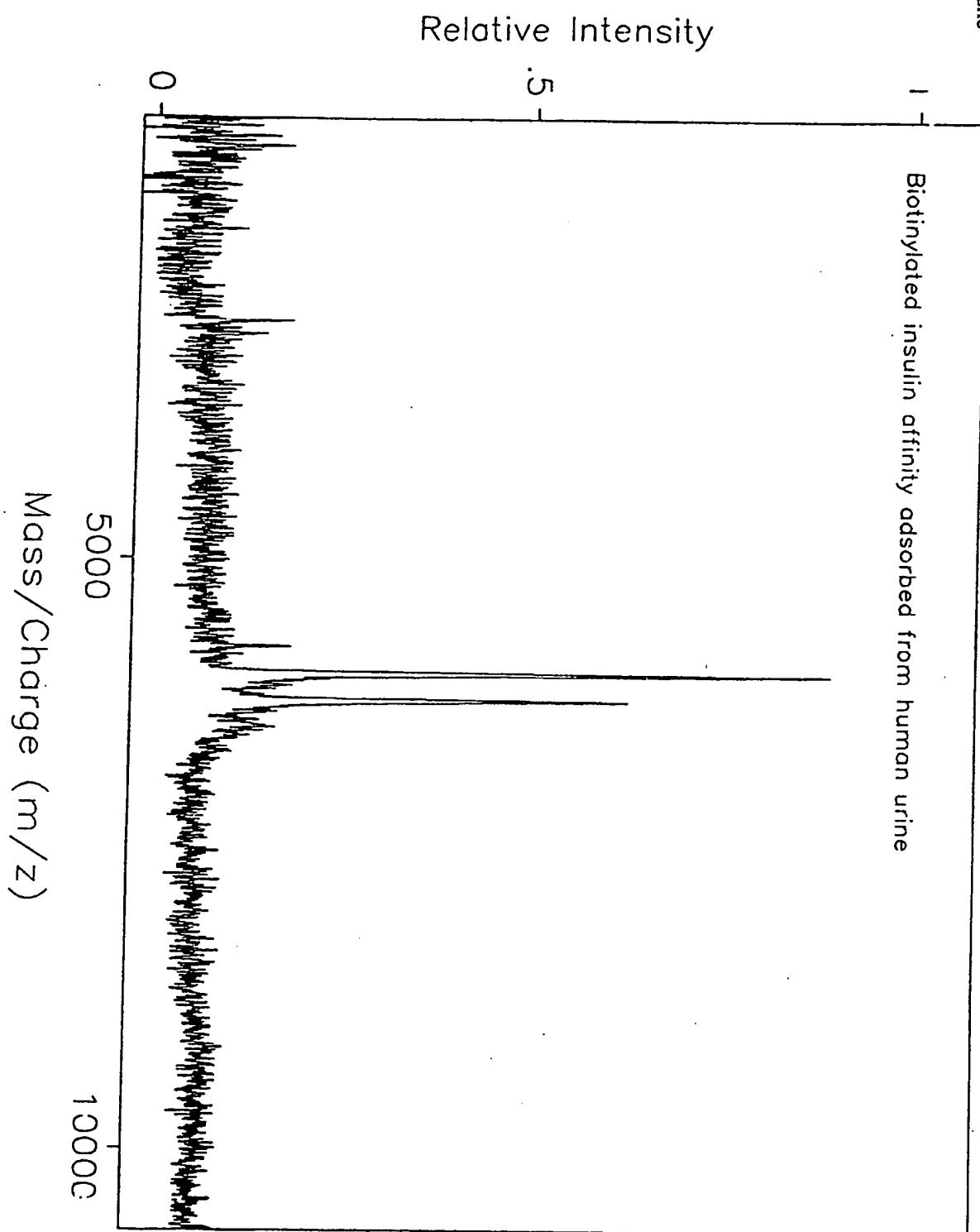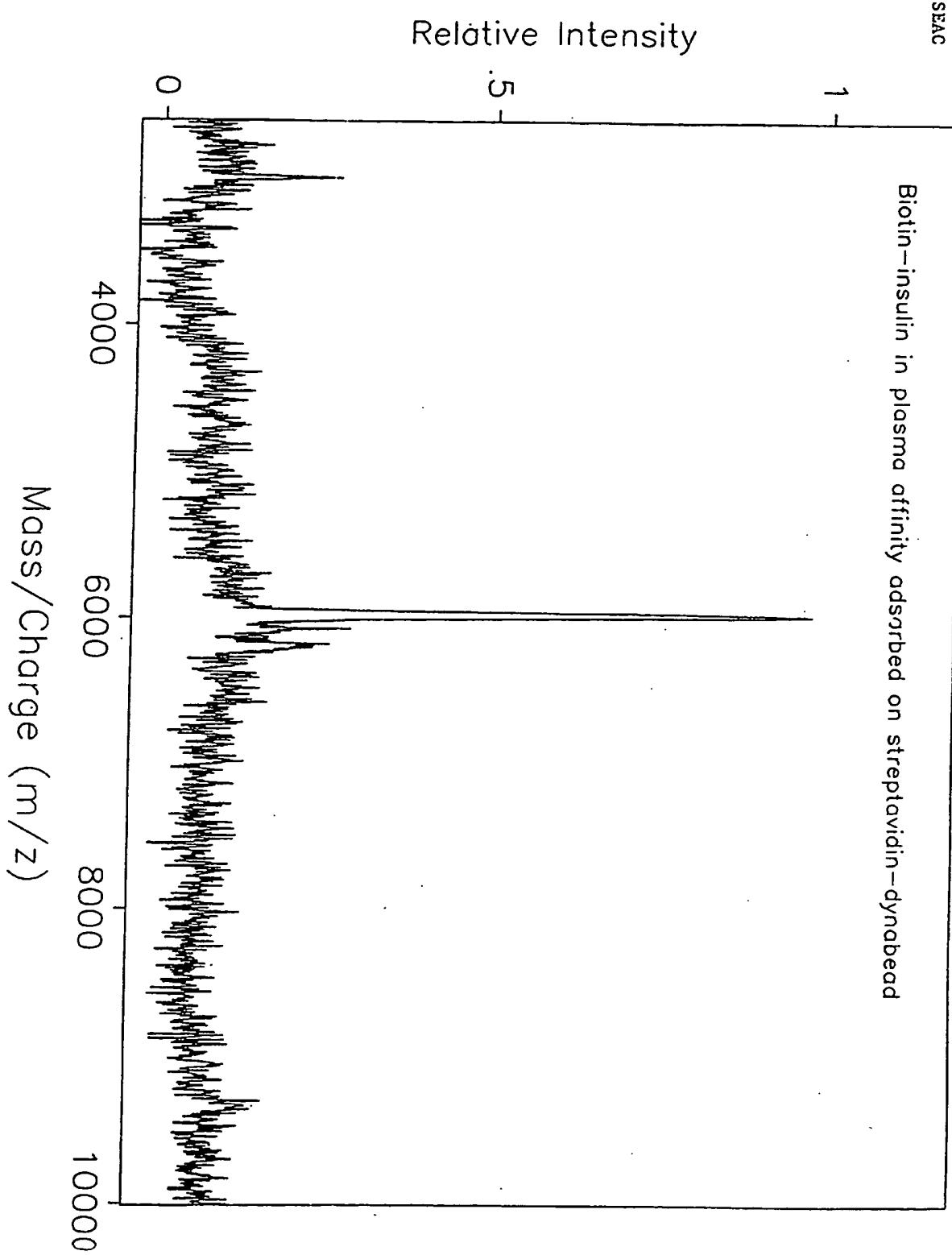
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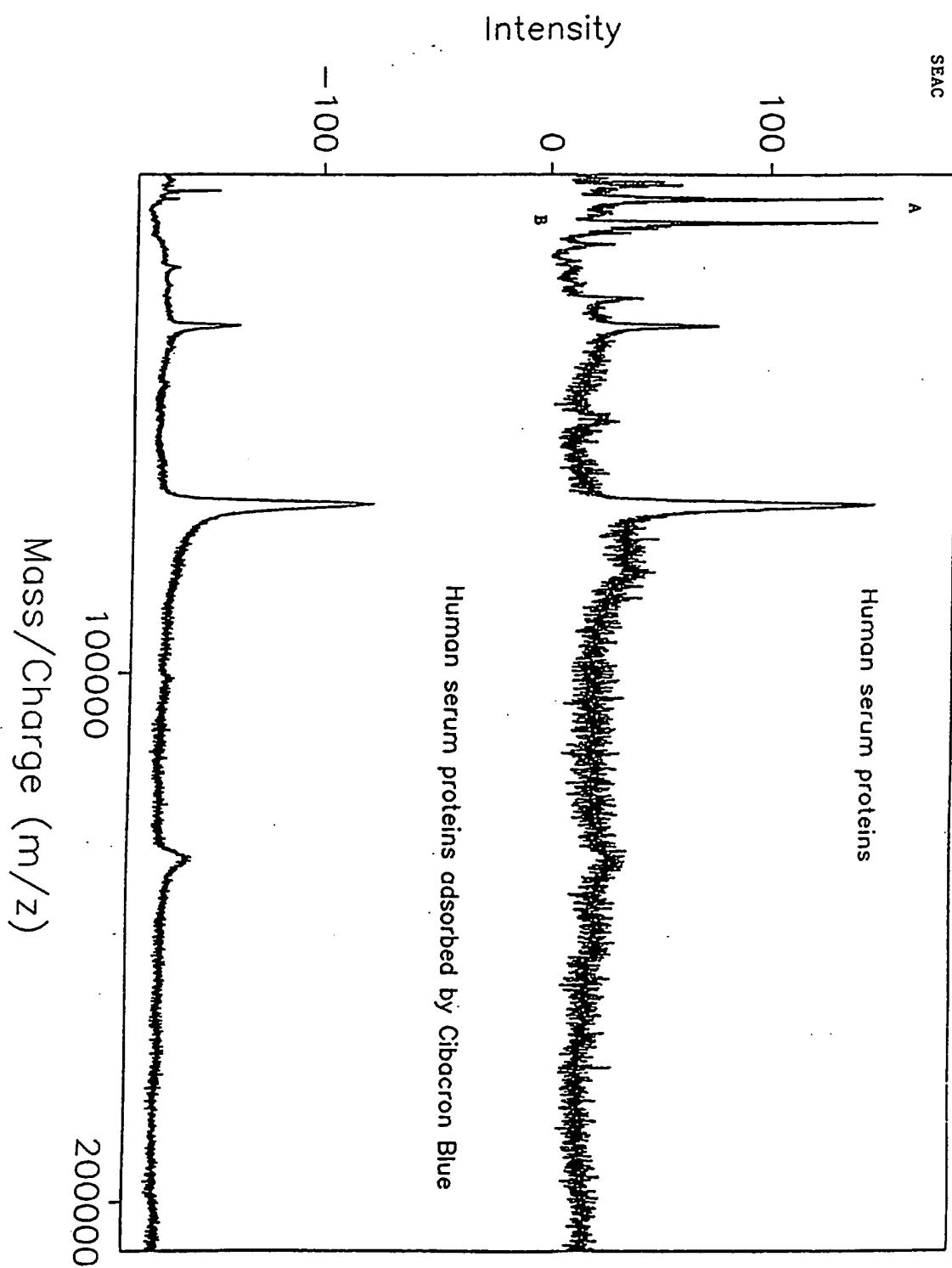
FIGURE 17B

FIGURES 18A & 18B

SEND
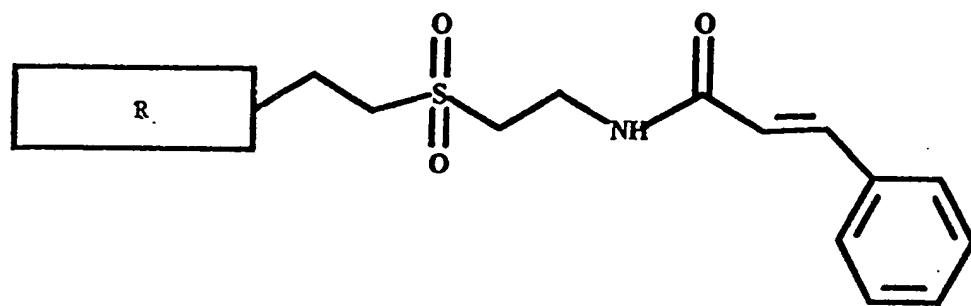


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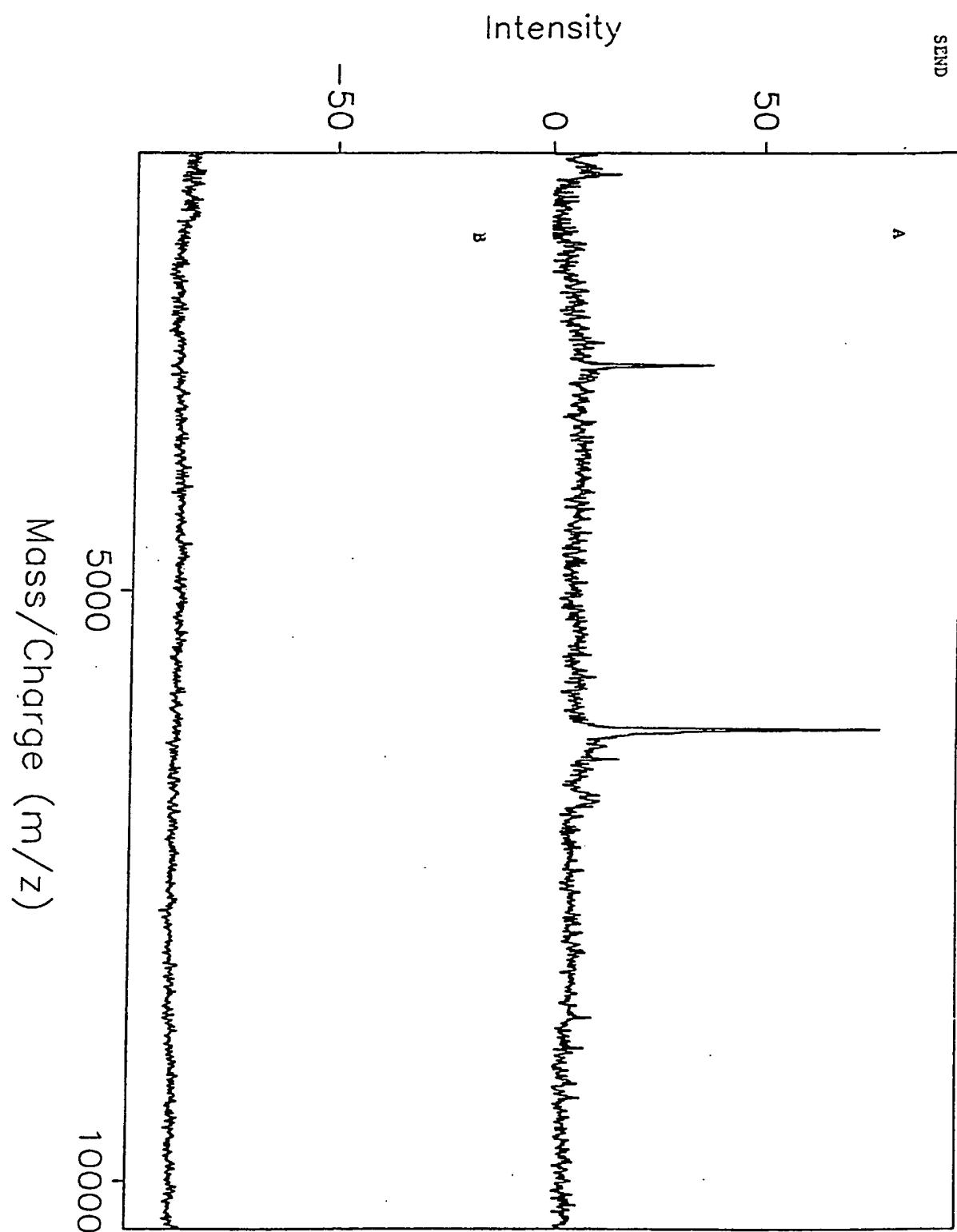
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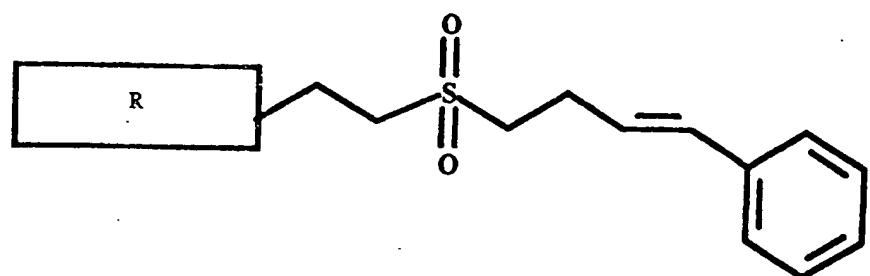
FIGURE 21
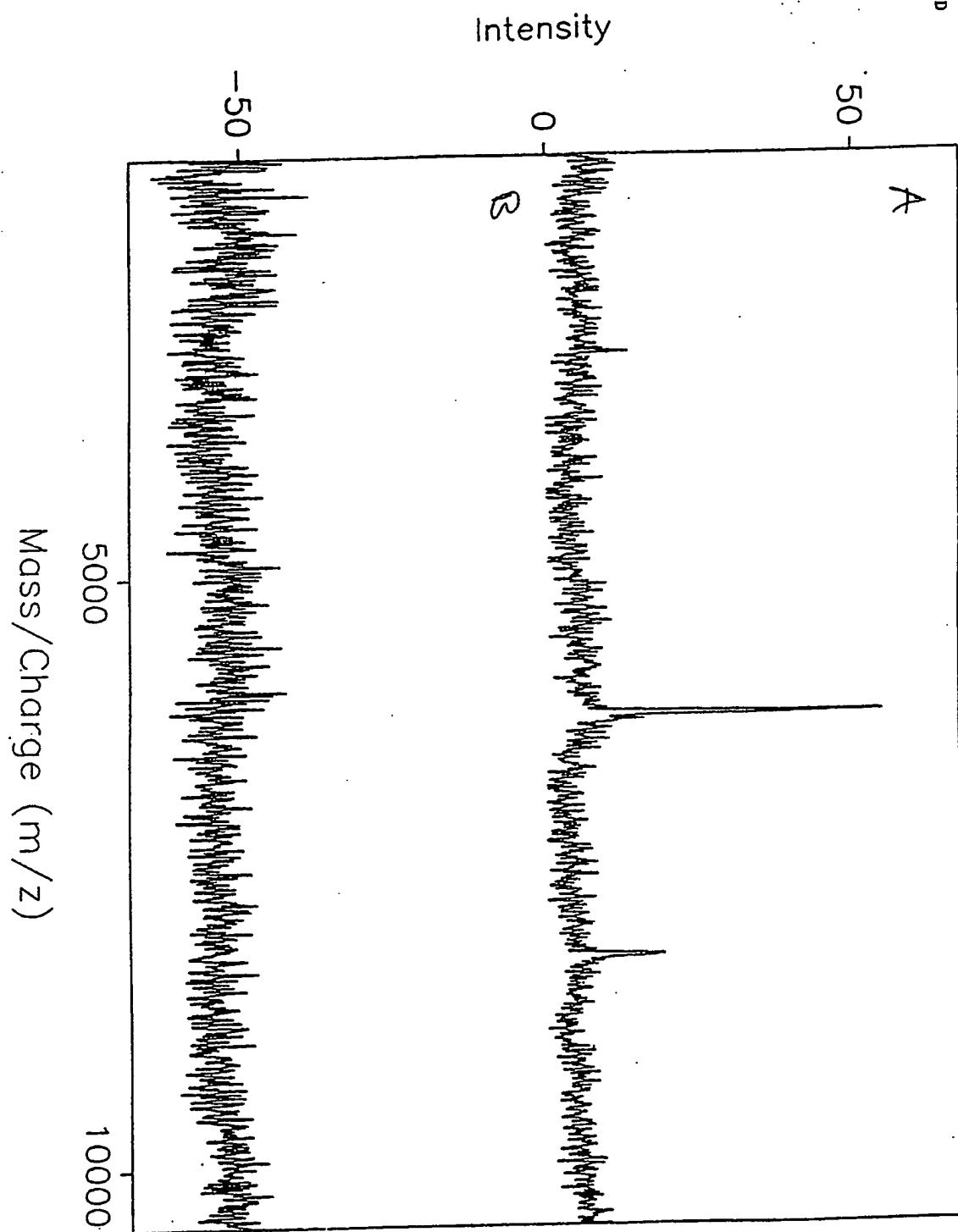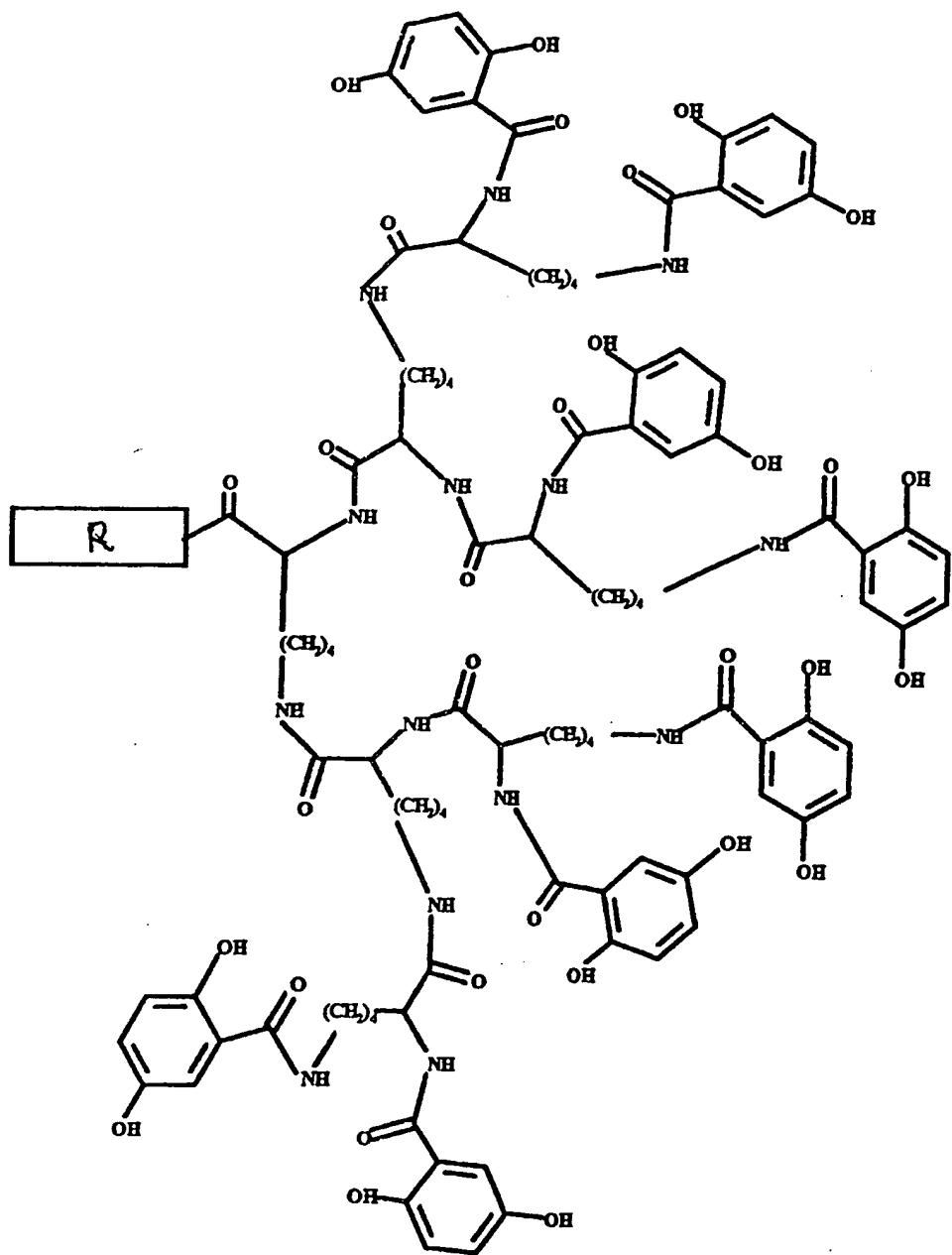
FIGURE 22

FIGURE 23
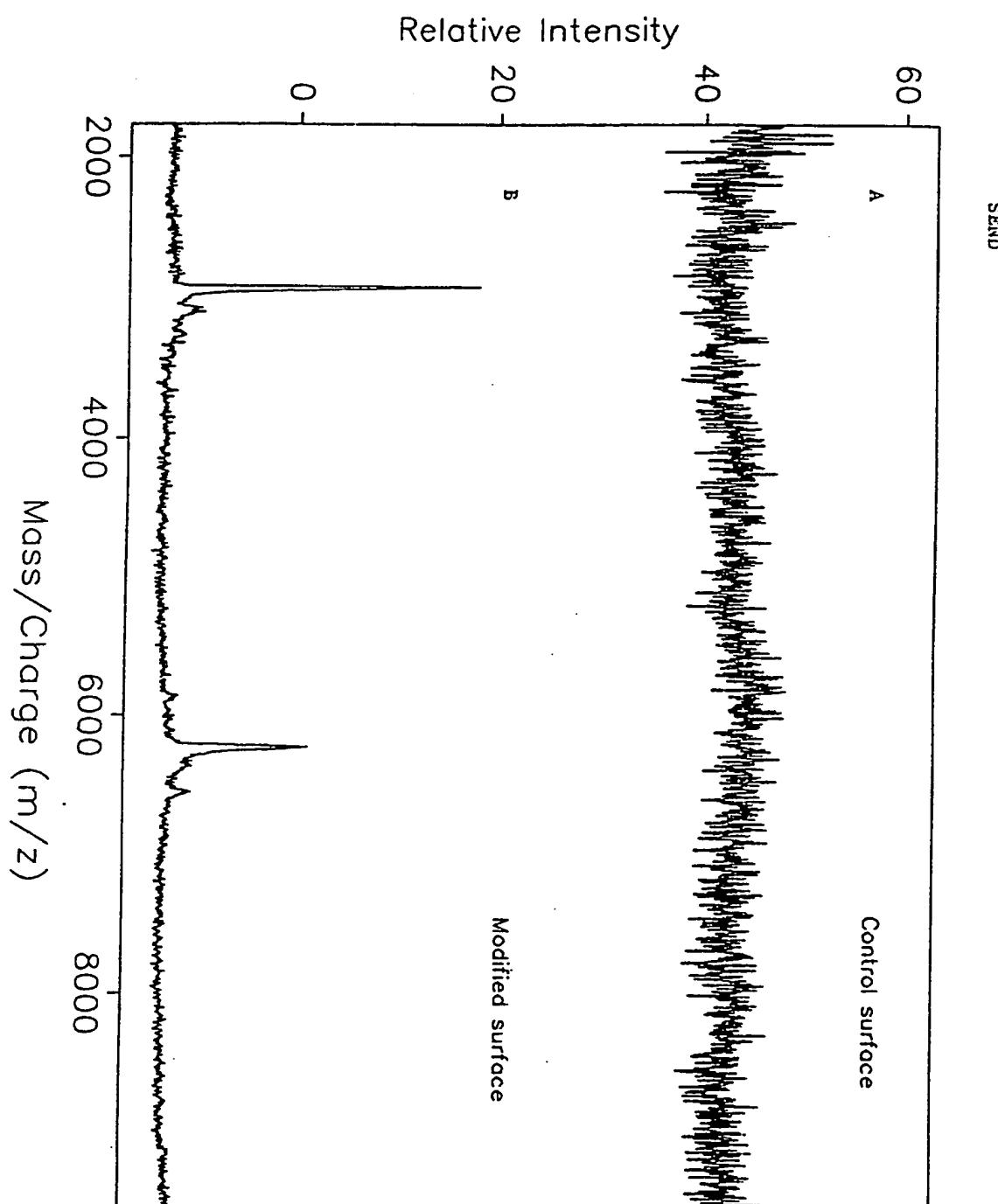
FIGURE 24
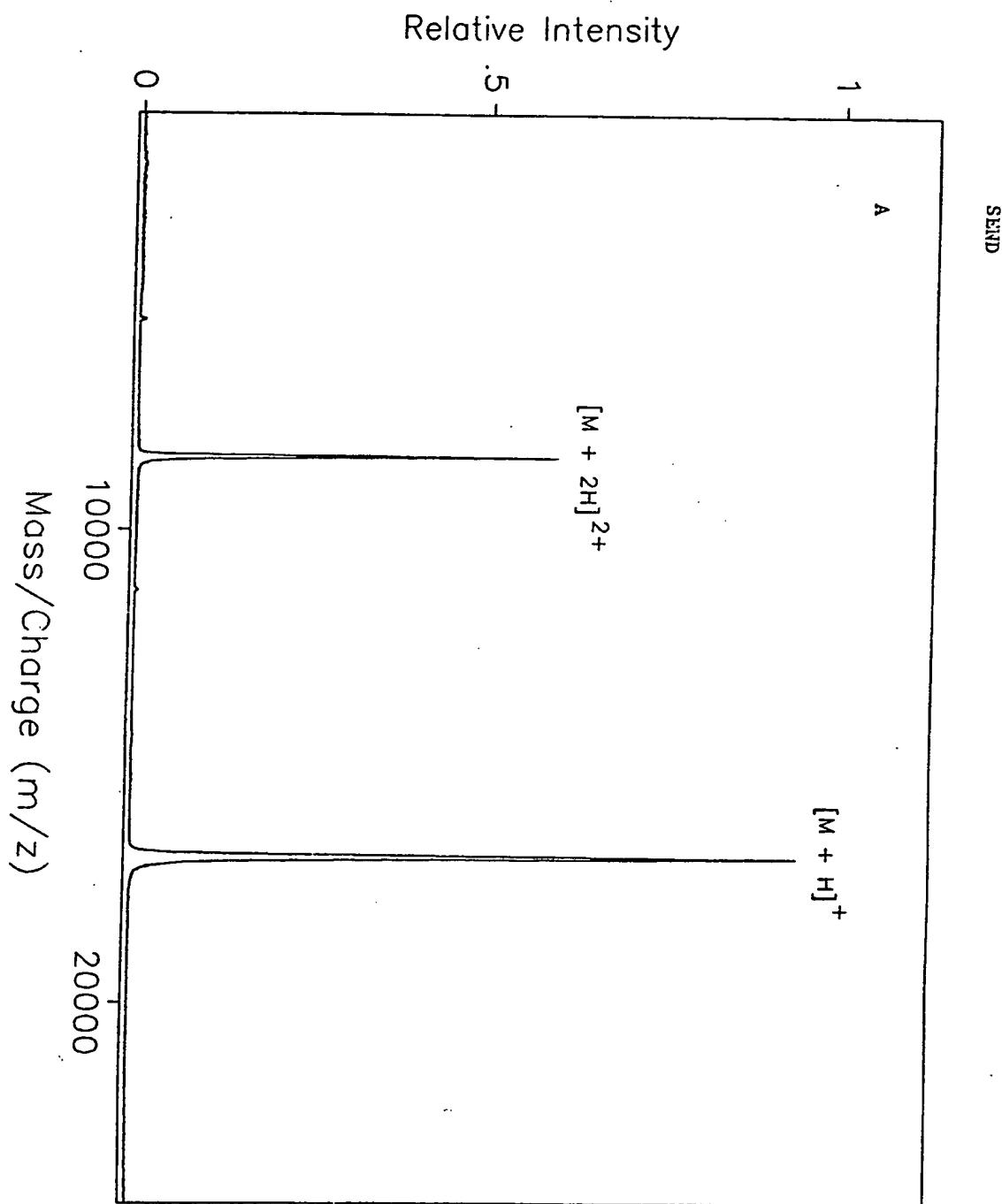
FIGURE 25A
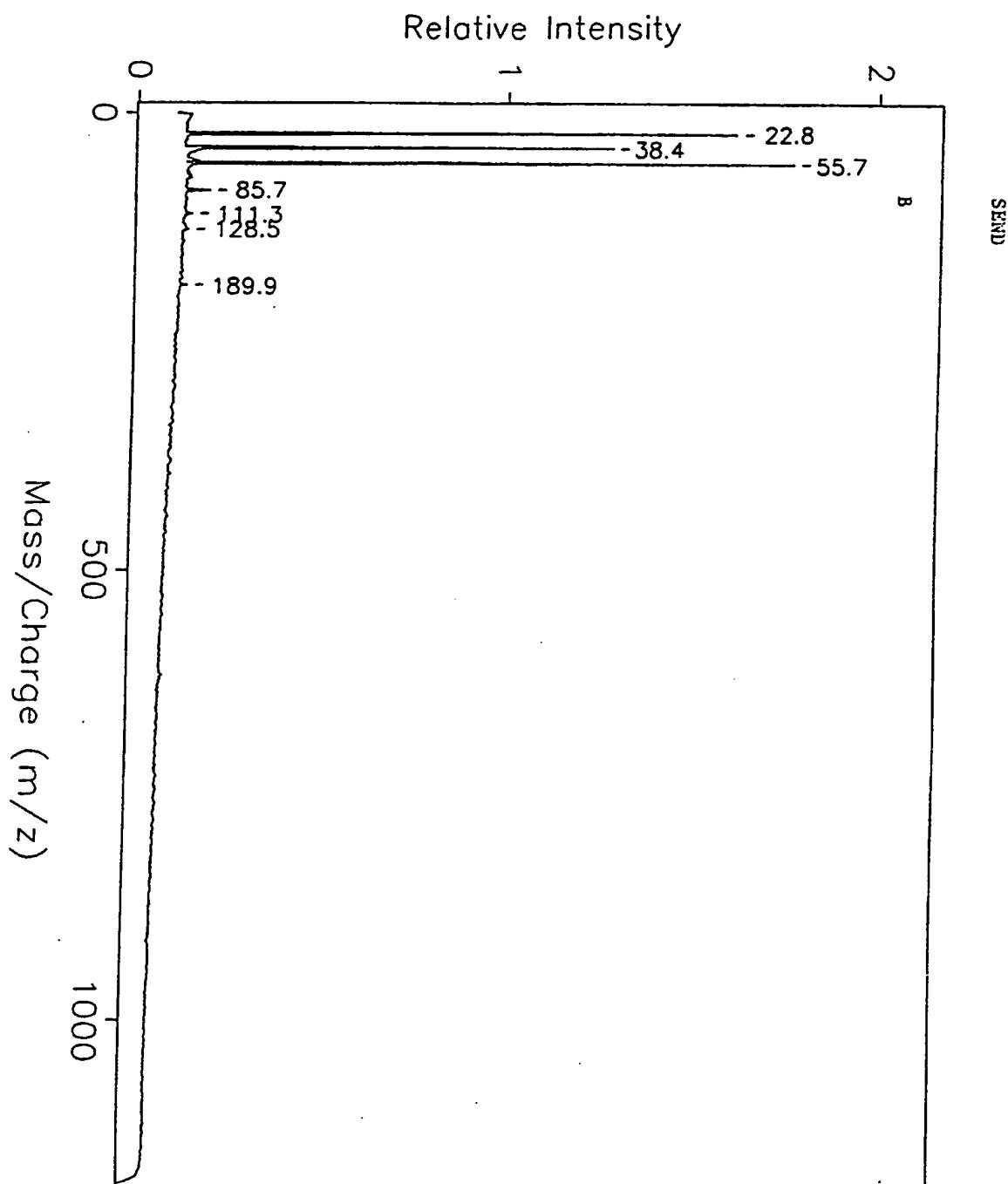
**FIGURE 25B**

SEND



Figure 26

SEND
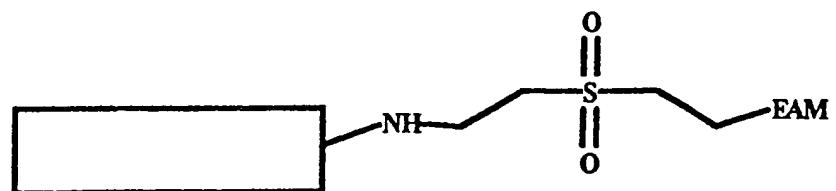


Figure 27

SEND
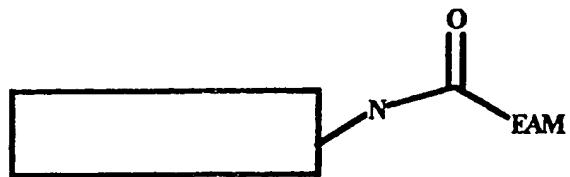


Figure 28

FIGURES 26, 27 & 28

SEND
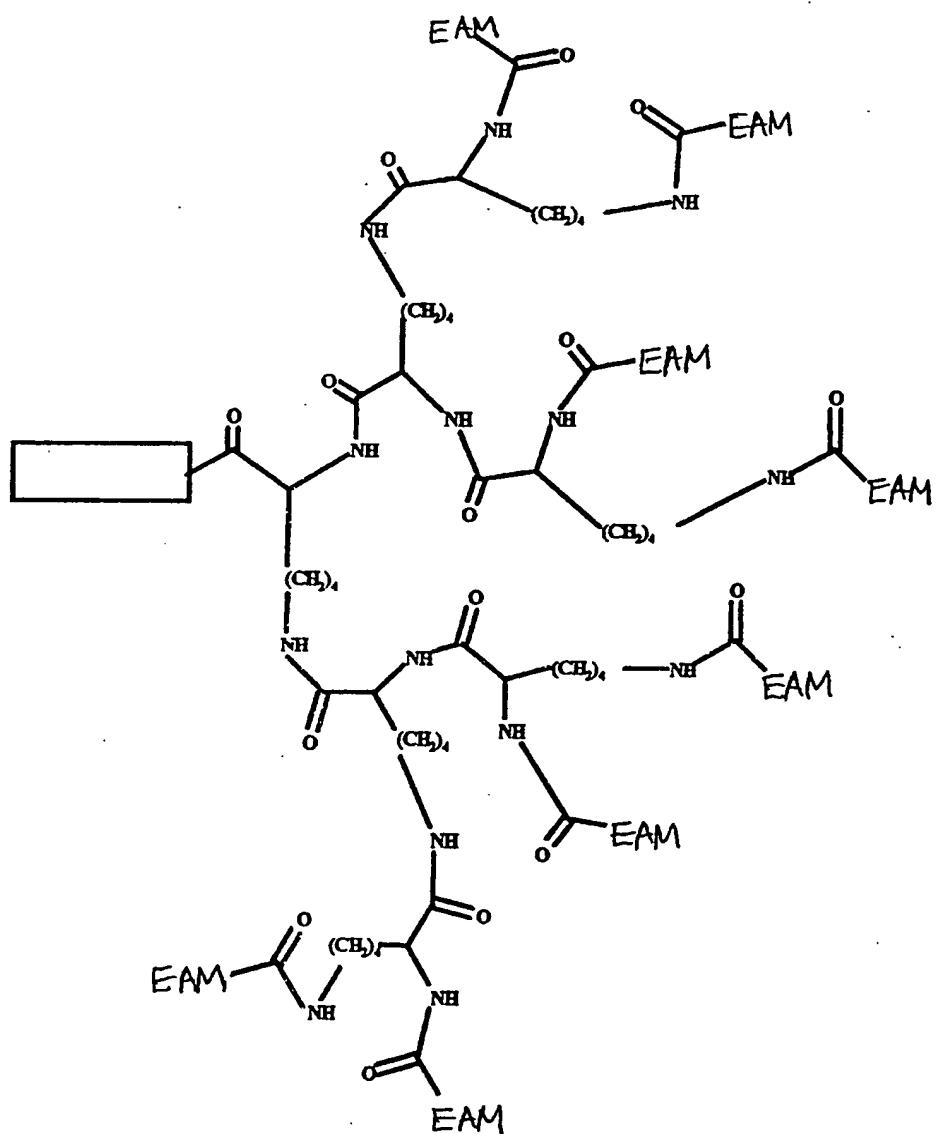


FIGURE 29

SEND



FIGURE 30

FIGURE 31

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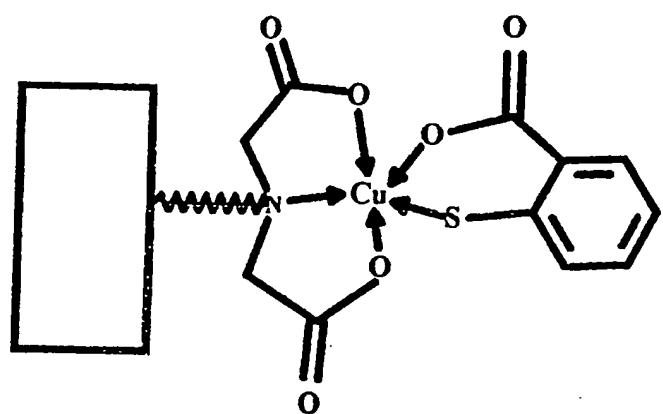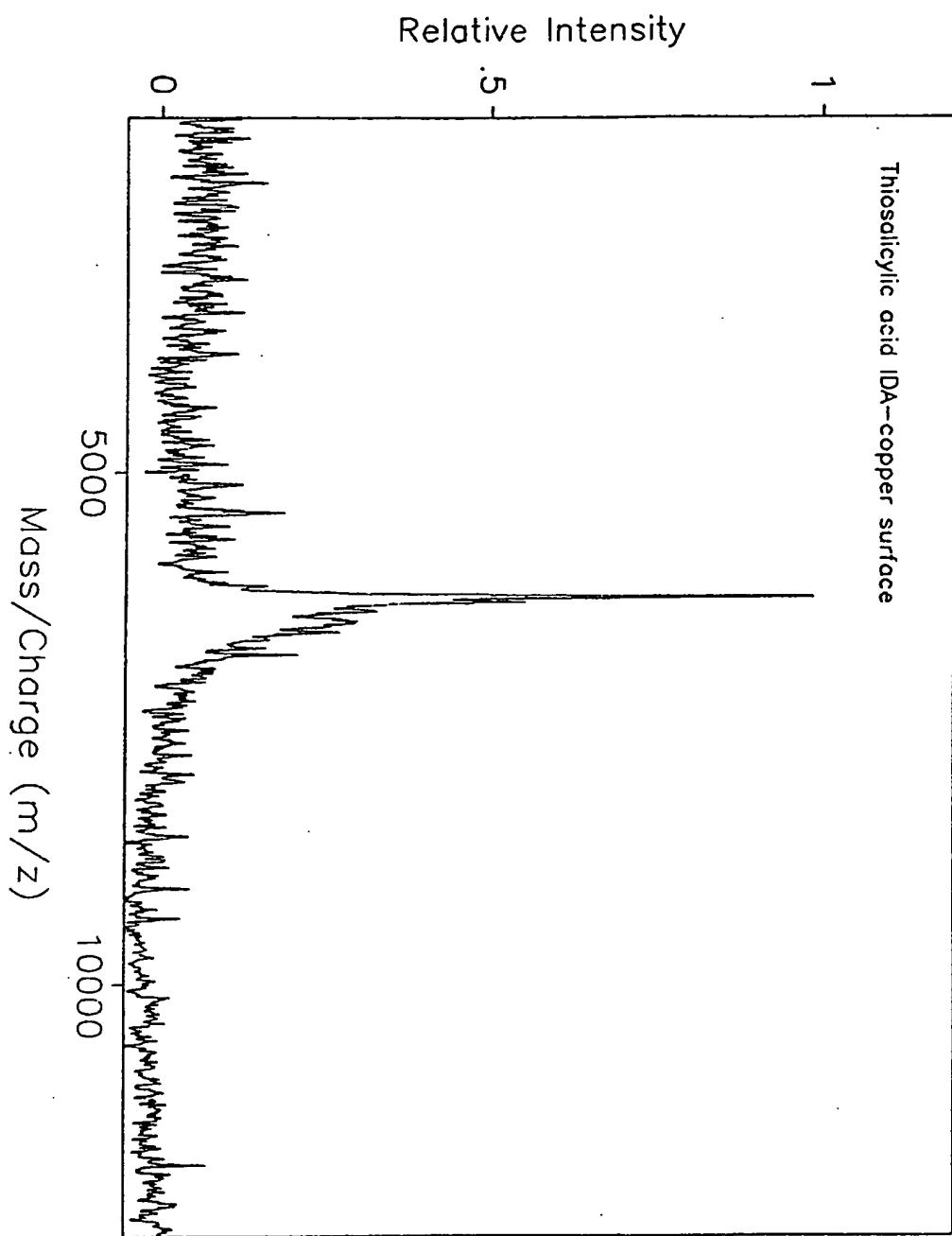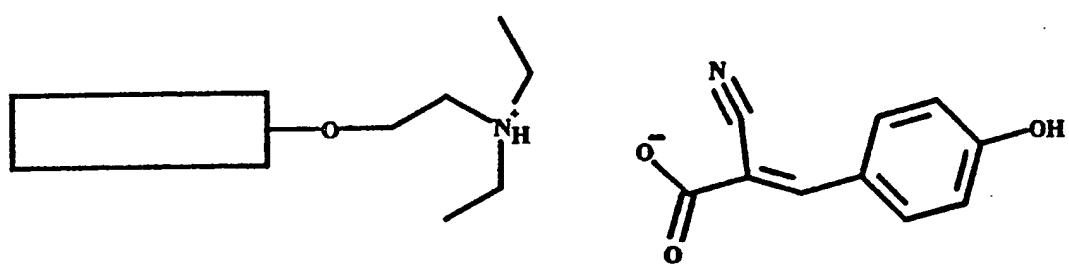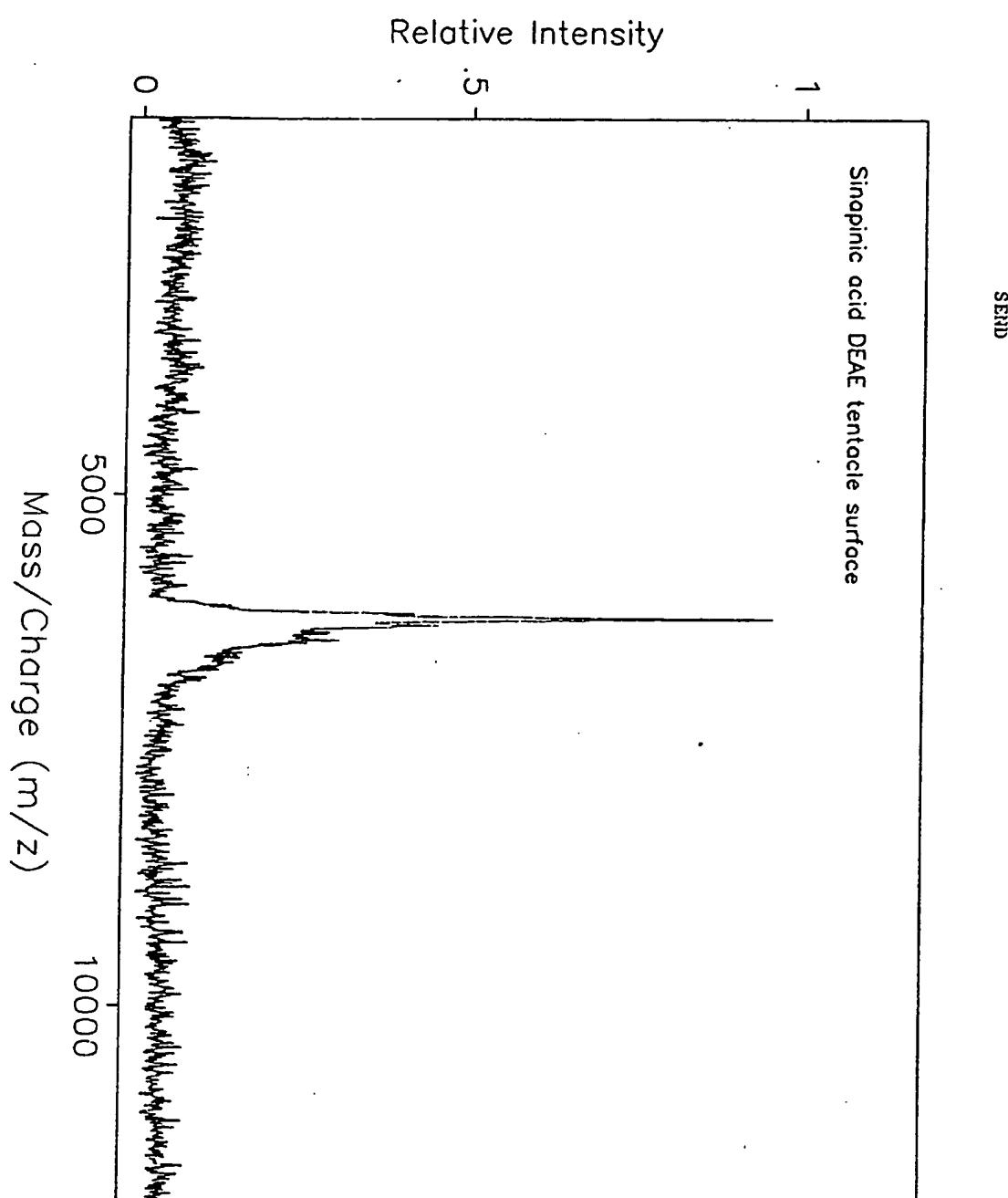
'SEND'



FIGURE 32

FIGURE 33A
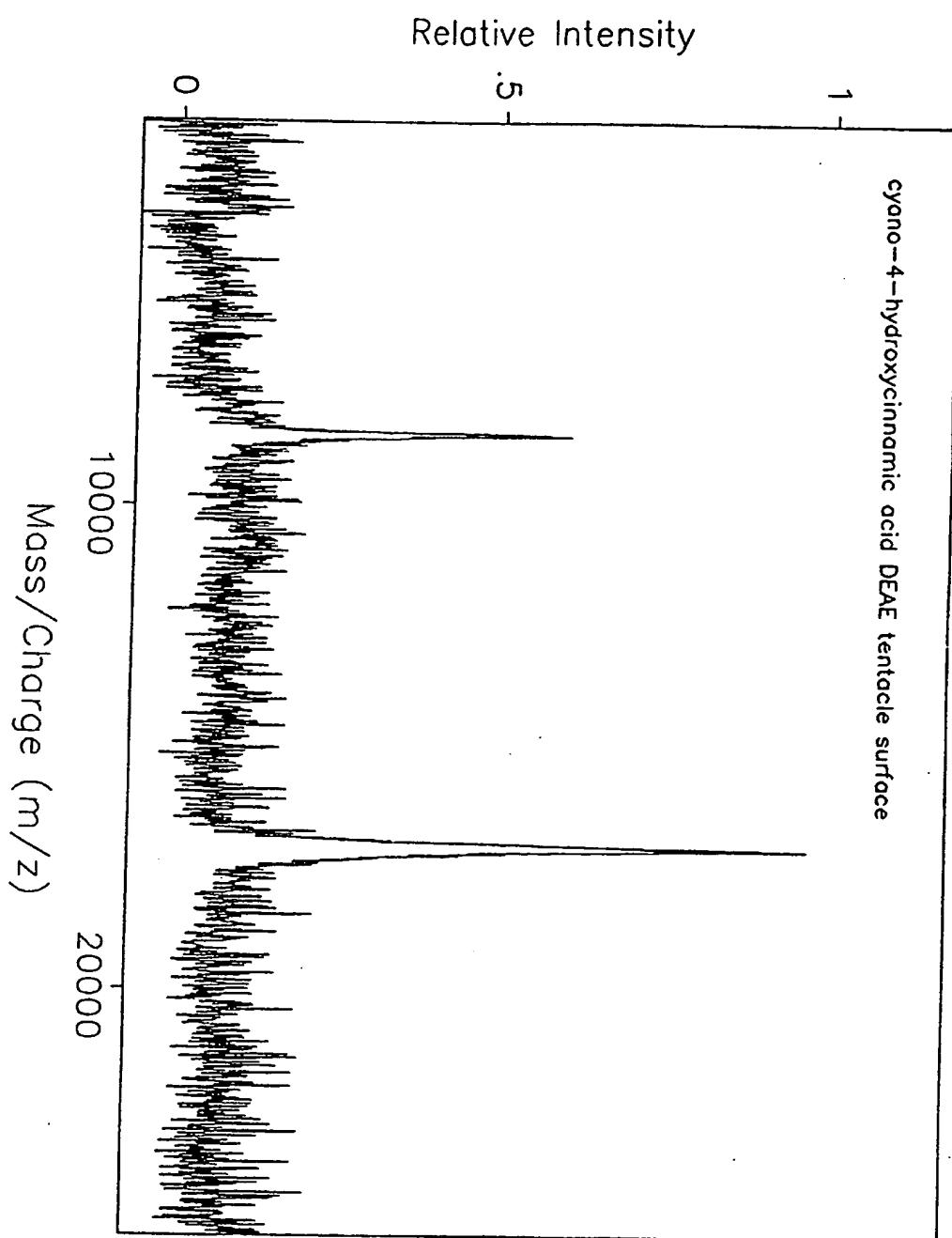
FIGURE 33B

SEND
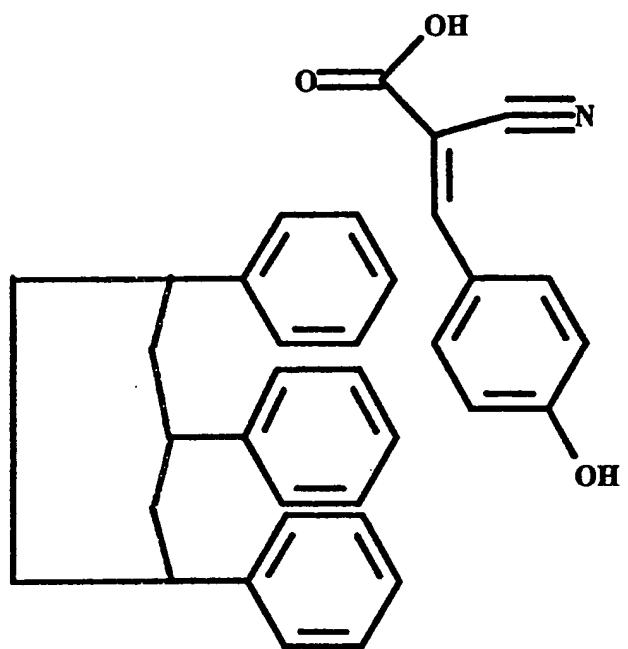


FIGURE 34
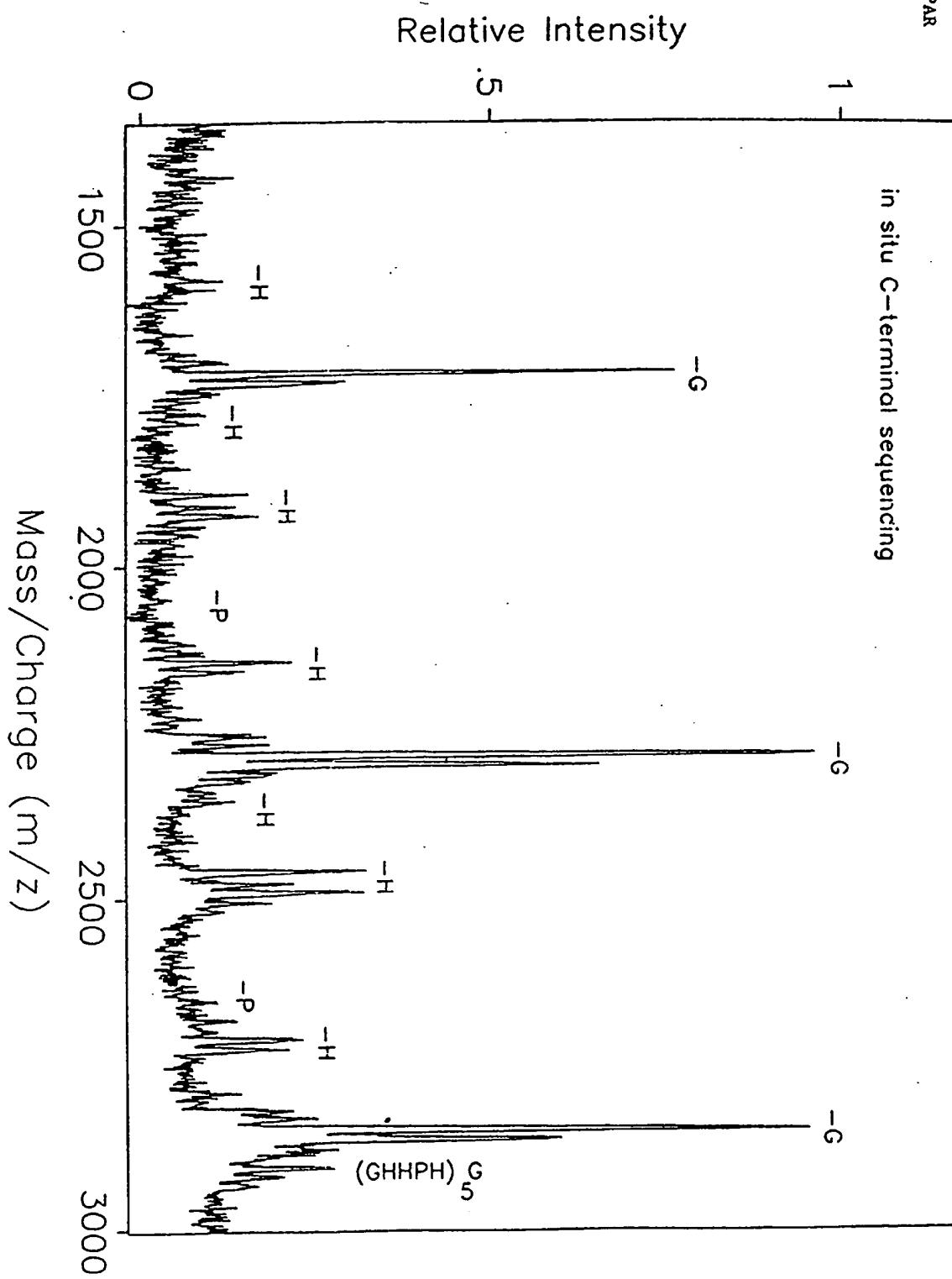
FIGURE 35

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# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/543
US CL : 436/173, 518; 250/287

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/173, 518, 178, 63, 155; 250/287, 288, 423

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US, A, 5,124,267 (HUMPEL ET AL) 23 JUNE 1992, SEE THE ENTIRE DOCUMENT. | 1-73 |
| Y | US, A, 5,209,919 (TURTELTAUB ET AL) 11 MAY 1993, SEE THE ENTIRE DOCUMENT. | 1-73 |
| Y | US, A, 4,686,366 (STUKE) 11 AUGUST 1987, SEE THE ENTIRE DOCUMENT. | 1-73 |

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

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|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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| "E" | earlier document published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
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| "P" | document published prior to the international filing date but later than the priority date claimed | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 08 SEPTEMBER 1994 | 16 SEP 1994 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br>LYLE A. ALEXANDER |
| Facsimile No.    (703) 305-3230 | Telephone No.    (703) 308-0196 |

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